OB03

A Novel Supercritical CO_2-Based Decellularization Method for Maintaining Scaffold Hydration and Mechanical Properties

Michael A. Matthews[a,b], Dominic M. Casali[a], Rachel M. Handleton[b]
[a]Department of Chemical Engineering, University of South Carolina, Columbia, SC 29208, USA
[b]Biomedical Engineering Program, University of South Carolina, Columbia, SC 29208, USA
*matthews@cec.sc.edu

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

Presented herein is a novel $scCO_2$ decellularization method that preserves matrix hydration state and mechanical properties. Water in the native tissue is maintained by presaturating $scCO_2$ with water; this was confirmed by experiments on two model scaffolds. Complete decellularization of porcine aorta was not attained with $scCO_2$ and additives alone. Subsequently, a hybrid method was discovered that combines briefly exposing the tissue to an aqueous detergent, followed by exposure to presaturated $scCO_2$. The hybrid method fully decellularized the tissue, as confirmed by histology and DNA quantification. This hybrid treatment was faster than the standard method, while preserving tissue structure and mechanical properties.

INTRODUCTION
Over 8,000 Americans die annually while awaiting an organ transplant, and currently over 120,000 Americans are on the national waiting list. Furthermore, the average wait time to obtain an organ transplant is several years. One way to address this problem is by replacing damaged tissues and organs with ones created by tissue engineering (TE), which could greatly reduce transplant wait times and also alleviate the current dearth of available organ donors.
Whether derived from synthetic or natural materials, TE scaffolds must be sterile, porous, mechanically strong, biocompatible, and of appropriate stiffness and surface chemistry for the application at hand [1]. Additionally, scaffold fabrication can introduce numerous mechanical and biochemical deficiencies, including loss of mechanical strength, loss of surface activity, denaturation of extracellular matrix (ECM) proteins, scaffold dehydration, and residually cytotoxic solvents, detergents, and/or crosslinking agents [2]. All of these challenges require novel and innovative TE scaffold fabrication methods to be continually developed and refined.

Additionally, TE scaffolds must direct cell proliferation and differentiation during tissue growth. This is a particular strength of naturally-derived biomaterials, which have been shown to promote constructive remodeling during tissue growth [3]. In particular, scaffolds prepared from decellularized tissues are uniquely able to receive and transmit signals to cells. Acellular ECM

scaffolds have also been shown to elicit an anti-inflammatory immune response, which may reduce risk of immune rejection [4].

Decellularization is accomplished using a variety of different techniques, including physical, chemical, and enzymatic treatment [5]. Treatment with aqueous detergents, such as sodium dodecyl sulfate (SDS) and Triton X-100, is most common. Detergents lyse cell and nuclear membranes, but also denature proteins, which can disrupt glycosaminoglycans (GAGs), growth factors, and ECM ultrastructure [6]. Because of these hazards, it has become common for protocols to use detergents at very low concentrations over several days or even weeks, minimizing ECM damage while eventually removing all cells. Though this approach is 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.

One relatively unexplored method is treatment employing supercritical carbon dioxide ($scCO_2$). $scCO_2$ is non-toxic, non-flammable, and relatively inert. Its mild critical temperature (31.1°C) makes it viable physiologic temperatures, and it has desirable transport properties such as high diffusivity, relatively high density, and low viscosity [7]. $scCO_2$ has been used extensively in TE applications that involve synthetic materials, particularly in polymer foaming, where CO_2 is used to fabricate TE scaffolds from synthetic polymers. $scCO_2$ has also been utilized in other biomedical applications, including extraction of biologically-relevant molecules, critical point drying, pasteurization, and sterilization of biomaterials and medical devices [8].

A novel decellularization technique using $scCO_2$ may give considerably faster decellularization, on the order of hours instead of days. The absence of harsh chemicals or solvents could also mitigate damage to the ECM. In 2008, Sawada *et al*. used $scCO_2$ to decellularize porcine aorta, but dehydration of the scaffold during treatment prevented further progress [9]. In previous research, our group presented a method, presaturation of $scCO_2$ with water, that greatly reduces tissue dehydration during $scCO_2$ treatment [10].

Our broad aim is to develop a novel $scCO_2$ decellularization method that also maintains the hydration state of the treated tissue. The objectives of this study are: (1) to quantify the extent of decellularization in porcine aorta using $scCO_2$ with different additives, pretreatments, and thermodynamic conditions; and (2) to present a hybrid detergent/$scCO_2$ treatment that decellularizes the tissue more quickly and as effectively as a standard detergent treatment. Achieving these objectives will enable further development of CO_2-based decellularization for TE scaffolds.

MATERIALS AND METHODS

Procurement and Standard Detergent Treatment

Porcine aorta was obtained from a local abattoir, rinsed with phosphate-buffered saline (PBS) and cut into ring-shaped sections measuring about 1 cm in length. Specimens were stored at -20°C for up to 48 hr until being treated with $scCO_2$ or a standard SDS treatment. The standard SDS treatment was performed according to the protocol of Funamoto *et al.* [11]. Tissues were pretreated for 1 hr in a solution containing 0.2% (w/v) EDTA and 10 mM pH 8 Tris buffer to increase cell membrane permeability. They were then decellularized for 48 hr under light agitation in 0.1% (v/v) SDS, 10 mM Tris buffer, 0.2 mg/mL DNase I, and 0.02 mg/mL RNase A.

Specimens were washed with PBS several times over the course of 24 hr to remove cell debris and residual detergent; thus the total time required for decellularization was 72 hours.

Decellularization with Supercritical CO_2

The aorta specimen (8) was loaded into the treatment chamber (7) of the $scCO_2$ apparatus, shown in Figure 1. The apparatus contained valves and fittings (2) rated for high pressures up to 68.9 MPa. Liquid carbon dioxide (1) was compressed in a chilled syringe pump (3) and slowly bubbled into the presaturation chamber (5) to maximize mass transfer. In this chamber, the additive and $scCO_2$ were stirred vigorously until reaching thermodynamic equilibrium (10-15 min with water and water solutions, 1-2 min for pure ethanol). Two different additive solutions were used to determine whether aqueous additives enhanced decellularization: ethanol and water/ethanol.

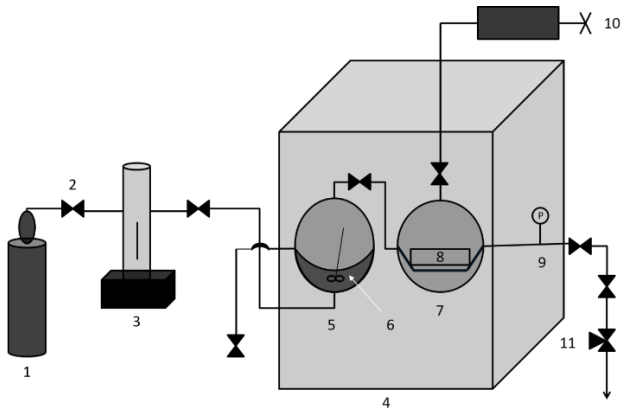

Figure 1. $scCO_2$ Apparatus

Once equilibrium was reached, the valve to the treatment chamber was opened, and $scCO_2$ flow was programmed to 1 mL/min at the pump inlet. During treatment, the environmental chamber (4) was used to maintain the temperature at either 10 or 37°C, and a back-pressure regulator (11) was used to keep the pressure of the $scCO_2$ in the vessels constant at either 10.3 or 27.6 MPa (1500 or 4000 psi, respectively). A manual hand pump (10) was used to depressurize the system at a rate of 0.34 MPa/min (50 psi/min).

Histology

After treatment, tissues were fixed in 10% neutral buffered formalin for at least 24 hr and embedded in paraffin. Tissues were then cut into 5 μm sections using a microtome and deparaffinized. The tissues were stained with either hematoxylin and eosin (to evaluate decellularization) or Masson's trichrome (to evaluate protein properties). A coverslip was mounted on slides, which were then viewed using a light microscope after waiting at least 24 hr for the slides to dry.

DNA Quantitation

DNA was quantified using the Invitrogen DNAzol reagent kit (Invitrogen, Carlsbad, CA). Optical density was measured at 260 nm using a spectrophotometer (DU 730 model, Beckman-Coulter, Brea, CA) and the DNA concentration was calculated based on the absorbance measurement and initial mass of the tissue.

Hybrid SDS/CO_2 Treatment
After analyzing the results of the above treatments, development of a hybrid detergent/$scCO_2$ treatment was desired. The hybrid treatment involved exposure of tissue to the standard detergent treatment solution, followed by 1 hr $scCO_2$ treatment described previously in lieu of the PBS wash. Water and ethanol were used together as additives.

Mechanical Testing
The mechanical properties of treated aorta specimens were quantified using a uniaxial ring test as described by Twal *et al*. [12]. Annular samples were mounted onto a Bose Electroforce 3230 Biomechanical Tester using two parallel cannulas. Specimens were subjected to three preconditioning cycles at a rate of 0.05 mm/s with a maximum stretch ratio of 1.2 during each cycle. Samples were kept moist with PBS during preconditioning to prevent dehydration. At the start of the identical fourth cycle, load and displacement data were recorded at a rate of 50 points/sec using Wintest software.

Residual SDS Quantitation
Residual SDS from the standard and hybrid treatments was quantified using an SDS Detection and Estimation Kit (G Biosciences, St. Louis, MO).

Statistical Analysis
Numerical data are presented as mean values plus or minus one standard deviation. A Student's *t*-test was used to analyze confidence in statistical differences between groups.

RESULTS
The objective of decellularization is to maximize removal of cells and cellular debris while minimizing alteration done to the ECM during treatment [6]. Currently, there is no universally-accepted standard for evaluating the extent of decellularization. This is not surprising, because tissues vary greatly in stiffness, cell density, ECM composition, and numerous other characteristics. Therefore, decellularization processes must be tailored to the specific tissue of interest. However, Badylak's group recently proposed a list of three criteria that can adequately describe a decellularized tissue of any kind. They are [5]:

1. Lack of visible nuclear material in H&E and/or DAPI-stained sections
2. Total amount of double-stranded DNA less than 0.05 μg/mg dry tissue
3. No individual DNA fragment longer than 200 base pairs

In this study, we focused on the first two criteria by performing H&E staining and DNA quantitation on porcine aorta after $scCO_2$ treatment. Treatments included SDS treatment, treatment with dry $scCO_2$, and treatment with presaturated $scCO_2$ using ethanol and water/ethanol. The thermodynamic conditions chosen were based on the factorial design and process optimization performed in Sawada's work [9].

Tissue sections from each treatment were stained with hematoxylin and eosin (H&E) and observed under an optical microscope. SDS exposure indicated significant damage to ECM fibers and substantial damage to cell membranes. Tissues treated with dry CO_2 showed less ECM damage but mostly intact cells (no significant evidence of decellularization).

To increase the polarity of the $scCO_2$ mixture, two additional treatments were investigated, using ethanol and ethanol/water as additive solutions. Figure 2 displays H&E-stained sections from these treatments. Treatment with ethanol alone shows considerable shriveling and branching of the elastic fibers, as was expected. However, the use of ethanol does not aid considerably in cell removal. Though some of the areas where the ECM is damaged have fewer cells, the intact elastic fibers have numerous intact cells attached to them. The addition of water to the ethanol does not markedly change the extent of decellularization, but does significantly improve the condition of the elastic fibers. This finding is expected based; the primary objective of using water as an additive is to prevent dehydration, not to remove cells. Including water as an additive was notably more effective in maintaining the alignment of the elastic fibers than ethanol alone. This supports findings in our previous work, which showed that presaturating $scCO_2$ with water before treatment prevents dehydration of the ECM during $scCO_2$ treatment.

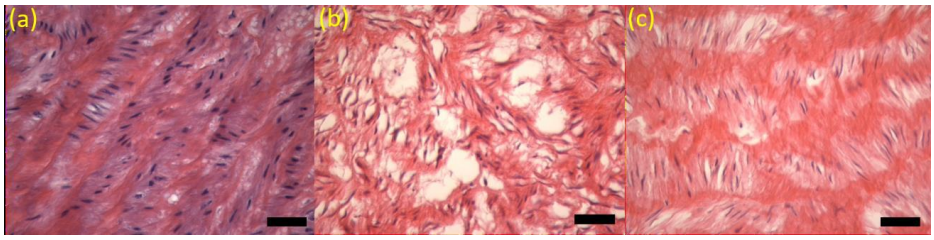

Figure 2. $scCO_2$ Treatments: (a) control; (b) $scCO_2$/ethanol; (c) $scCO_2$/water/ethanol

On the contrary, when ethanol is the only additive, shriveling and fraying of the ECM fibers is observed, as in Sawada's paper. These findings were also confirmed visually and by manual handling, as treatment clearly increased the rigidity of the matrix when water was not added, while the addition of water maintained the apparent flexibility and pliability of the material. Though interesting, the prevention of tissue dehydration is made impractical by the lack of cell removal in these experiments.

DNA quantitation confirmed histological observations. The failure of the $scCO_2$/ethanol mixture to decellularize is the most surprising result. Based on this, we theorized that $scCO_2$ in general may suffer from an inability to disrupt cell membranes. To test this hypothesis, a two-step hybrid SDS/$scCO_2$ decellularization treatment was investigated. With this treatment, tissues were treated with SDS as described previously, but without the subsequent PBS wash. Instead, tissues were then treated (washed) for 1 hr with $scCO_2$ presaturated with ethanol and water at the same thermodynamic conditions used previously.

The effect of the hybrid treatment can be seen in Figure 3. Using this hybrid approach (image c), there are no visible intact cells or cellular debris. Therefore, it is likely that the tissue is fully decellularized. This is an exciting and intriguing result that suggests polar supercritical CO_2 will extract intracellular debris, but cannot do so unless another agent is used to enhance membrane permeation or rupture. Significantly, the ECM fibers appear to be intact and mostly undisturbed compared to the complete SDS treatment (image b). Maintenance of fiber integrity may be a result of shorter exposure time to SDS; the $scCO_2$/water + ethanol "wash" takes only an hour instead of the day or more required for PBS washing. DNA quantitation confirmed this finding. This is a very exciting result, as the hybrid method is able to achieve the original objective: to decellularize effectively while avoiding dehydration of the tissue.

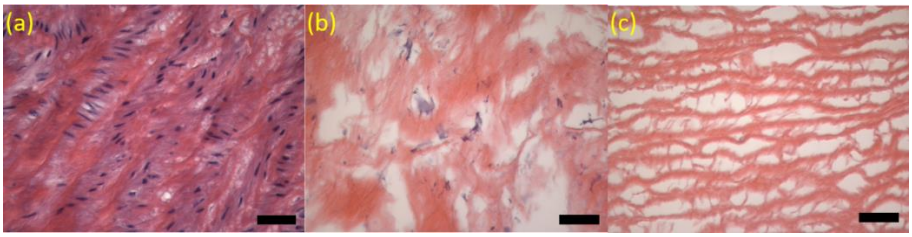

Figure 3. Hybrid Treatment: (a) control; (b) SDS only; (c) hybrid treatment

To analyze the physical properties of treated aorta, uniaxial tensile testing was performed on samples from selected treatments (SDS, CO_2/ethanol, and hybrid). Stress responses for each treatment are presented in Table 1. As expected, SDS exposure denatures proteins and reduces stiffness, while treatment with $scCO_2$ and ethanol causes a significant increase in stiffness because of dehydration. Both of these extremes are mitigated by the hybrid treatment: faster treatment reduces protein denaturation and presaturation with water prevents matrix dehydration. These findings were confirmed by trichrome staining.

Table 1 – Uniaxial Ring Test Stress at $\lambda = 1.1$

Treatment	Stress (kPa)
None (Native Tissue)	32.7 ± 5.4
SDS	8.9 ± 0.9**
$scCO_2$/ethanol	60.6 ± 13.5*
Hybrid	21.9 ± 3.2*

*$p < 0.05$ compared to native
**$p < 0.01$ compared to native

Removal of SDS is another consideration for scaffold viability, as cytotoxicity is observed for many cell types at concentrations greater than about 0.002% SDS [13]. Residual SDS was quantified for the standard SDS treatment and the SDS/$scCO_2$ hybrid treatment. It was found that that one hour of $scCO_2$ treatment removes about as much SDS as 24 hours of washing with PBS, a significant time savings. PBS washes also have diminishing returns, making the wash step last several days in many protocols to reduce SDS below cytotoxic levels. Thus, $scCO_2$ could compare even more favorably over longer time periods. This finding also indicates some solubility of SDS in the $scCO_2$ treatment solution; solubility may be low because SDS is a charged molecule, but SDS does have similar molecular weight to other molecules extracted by $scCO_2$ in other studies.

CONCLUSION

We demonstrated a novel $scCO_2$ method for decellularizing porcine aorta without compromising its hydration state. This method offers considerably faster decellularization of tissues without requiring long-term exposure to detergents or organic solvents. As anticipated, nonpolar $scCO_2$ solutions were proven ineffective for decellularizing porcine aorta, as measured by both histology and DNA quantitation, though presaturating $scCO_2$ with water did better maintain the hydration state of the matrix, even in the presence of other additives. More surprisingly, the addition of ethanol to increase $scCO_2$ polarity did not substantially intensify the extent of decellularization, suggesting that $scCO_2$ alone is unable to lyse the cell membrane and that the previously proposed mechanism of whole-cell extraction is unlikely to be valid.

The inability of $scCO_2$ alone is unable to disrupt cell membranes was further tested by the development of a hybrid decellularization protocol that utilized an SDS pretreatment step before washing with $scCO_2$ and water plus ethanol as additives. This treatment shows that $scCO_2$ can extract intracellular material if the cell membrane is lysed beforehand. Complete decellularization was achieved using this method, which was about 24 hr faster than the standard method and maintained the hydration state of the native tissue. Mechanical response of ECM decellularized by the hybrid treatment was similar to that of the native tissue, most residual SDS was removed, and trichrome staining showed similarity in collagen and elastin fibers before and after treatment. Still, further study is required to determine the capabilities and limitations of this method and to fully assess the effects of decellularization on the mechanical and biochemical properties of the matrix.

ACKNOWLEDGEMENTS
The authors wish to acknowledge the assistance of the University of South Carolina (USC) School of Medicine for preparation of histological specimens and of USC professor Tarek Shazly for assistance with mechanical testing.

REFERENCES

[1] F. Berthiaume, T.J. Maguire, M.L. Yarmush, Tissue Engineering and Regenerative Medicine: History, Progress, and Challenges, in: J.M. Prausnitz (Ed.) Annual Review of Chemical and Biomolecular Engineering, Vol 2, Annual Reviews, Palo Alto, 2011, pp. 403-430.

[2] B.-S. Kim, I.-K. Park, T. Hoshiba, H.-L. Jiang, Y.-J. Choi, T. Akaike, C.-S. Cho, Design of artificial extracellular matrices for tissue engineering, Progress in Polymer Science, 36 (2011) 238-268.

[3] J.E. Reing, L. Zhang, J. Myers-Irvin, K.E. Cordero, D.O. Freytes, E. Heber-Katz, K. Bedelbaeva, D. McIntosh, A. Dewilde, S.J. Braunhut, S.F. Badylak, Degradation products of extracellular matrix affect cell migration and proliferation, Tissue Engineering Part A, 15 (2009) 605-614.

[4] B.N. Brown, R. Londono, S. Tottey, L. Zhang, K.A. Kukla, M.T. Wolf, K.A. Daly, J.E. Reing, S.F. Badylak, Macrophage phenotype as a predictor of constructive remodeling following the implantation of biologically derived surgical mesh materials, Acta Biomaterialia, 8 (2012) 978-987.

[5] P.M. Crapo, T.W. Gilbert, S.F. Badylak, An overview of tissue and whole organ decellularization processes, Biomaterials, 32 (2011) 3233-3243.

[6] T.J. Keane, I.T. Swinehart, S.F. Badylak, Methods of tissue decellularization used for preparation of biologic scaffolds and *in vivo* relevance, Methods, 84 (2015) 25-34.

[7] M.A. McHugh, V.J. Krukonis, Supercritical Fluid Extraction: Principles and Practice, Butterworth, Stoneham, Mass., 1994.

[8] J. Zhang, T.A. Davis, M.A. Matthews, M.J. Drews, M. LaBerge, Y.H.H. An, Sterilization using high-pressure carbon dioxide, Journal of Supercritical Fluids, 38 (2006) 354-372.

[9] K. Sawada, D. Terada, T. Yamaoka, S. Kitamura, T. Fujisato, Cell removal with supercritical carbon dioxide for acellular artificial tissue, Journal of Chemical Technology and Biotechnology, 83 (2008) 943-949.

[10] D.M.M. Casali, M.A., Treatment of hydrated biomaterials with supercritical carbon dioxide, in: AIChE Annual Meeting 2014, Atlanta, GA, 2014.

[11] S. Funamoto, K. Nam, T. Kimura, A. Murakoshi, Y. Hashimoto, K. Niwaya, S. Kitamura, T. Fujisato, A. Kishida, The use of high-hydrostatic pressure treatment to decellularize blood vessels, Biomaterials, 31 (2010) 3590-3595.

[12] W.O. Twal, S.C. Klatt, K. Harikrishnan, E. Gerges, M.A. Cooley, T.C. Trusk, B. Zhou, M.G. Gabr, T. Shazly, S.M. Lessner, R.R. Markwald, W.S. Argraves, Cellularized microcarriers as adhesive building blocks for fabrication of tubular tissue constructs, Annals of Biomedical Engineering, 42 (2014) 1470-1481.

[13] S. Cebotari, I. Tudorache, T. Jaekel, A. Hilfiker, S. Dorfman, W. Ternes, A. Haverich, A. Lichtenberg, Detergent decellularization of heart valves for tissue engineering: toxicological effects of residual detergents on human endothelial cells, Artificial Organs, 34 (2010) 206-210.