Compressed Carbon Dioxide for Decontamination of Biomaterials and Tissue Scaffolds

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ABSTRACT

Biomaterials must be both sterile and free of contaminants prior to use, and this is particularly critical for the next generation of implants based on tissue engineering. With increasing complexity of tissue engineering scaffolds and multifunctional devices, there is a need for new approaches to decontamination, i.e. cleaning, disinfection, and sterilization. This work presents our recent results on several aspects of decontamination of both metallic and polymeric biomaterials using liquid and supercritical CO_2 technology. We demonstrate the removal of a lubricant oil and *E. coli* endotoxins from titanium surfaces with compressed CO_2. In another application, high level disinfection of a model hydrogel contaminated with *S. aureus* has been achieved with supercritical CO_2. For sterilization, results are also presented on killing *B. pumilus* spores using supercritical CO_2 containing trace levels of hydrogen peroxide.

INTRODUCTION

Rapid development of biomedical materials and devices technology has challenged current cleaning, disinfection, and sterilization processes [1]. This is particularly true for environment-sensitive biopolymers such as tissue engineering scaffolds and multifunctional devices having porous or irregular surfaces, or multiple layers. Hence, there is a crucial need for the development of new decontamination techniques without introducing potential contamination and without compromising the functionality of the device.

A failure to successfully remove or sterilize bacteria leads to bacterial colonization and biofilm formation [2-4], which may harbor bacterial residuals as endotoxins [5]. Most common cleaning methods employed in hospital reprocessing centers include washing with water and detergents, or organic solvents [6-7]. However, sometimes these cleaning methods are not sufficient to completely eradicate residual endotoxins [8] to acceptably safe levels ($\leq$ 20 EU/device) [9]. The major disinfection and sterilization methods include steam autoclave, gamma and ultraviolet irradiation, and chemical methods such as ethylene oxide and hydrogen peroxide [10-11]. However, no single process is suitable for sterilizing all medical devices and all of them have posed significant drawbacks in specific biomedical applications [12-15].

This work concerns several aspects of cleaning, disinfection, and sterilization of both metallic and polymeric biomaterials using compressed carbon dioxide (CO_2) (liquid and supercritical). For cleaning purposes, compressed CO_2-based processes were evaluated in the removal of lubricant oil and *Escherichia coli* endotoxins from titanium (Ti) surfaces. The CO_2 disinfection process was studied on a selected hydrogel, poly (acrylic acid-co-acrylamide)

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potassium salt, inoculated with *Staphylococcus aureus*. For sterilization, supercritical (sc) CO_2 was evaluated in killing B*acillus pumilus* spores with trace amounts of additives such as water, ethanol, and hydrogen peroxide (H_2O_2).

MATERIALS AND METHODS

Cleaning Ti surfaces contaminated either with lubricant oil or endotoxins

Commercially pure Ti disks with smooth surfaces measuring 12 mm in diameter and 2.5 mm in thickness were used for both oil and endotoxins. For the oil removal experiments, Ti disks were contaminated with 0.5 μL of Vasco 1000 lubribcant oil (Blaser Swisslube Inc., Goshen NY). Three processes were employed and compared; (1) cleaning with sc CO_2, (2) cleaning with liquid detergent (positive control), and (3) no solvent/detergent (negative control). Sc CO_2 treatments were carried out in a 1 L pressure vessel (FC series, Pressure Products Industries, Warminster, PA) at 27.6 MPa and 40 ºC. The disks were attached to the disk holder, placed in the pressure vessel, and allowed to spin at 400 rpm for four hours. Negative control was also carried out in the pressure vessel but with no CO_2 to evaluate the removal with spinning. Cleaning experiments for the positive control consisted in manual washing of the disks with liquid detergent (Extran 300 from EMD Chemicals Inc., Gibbstown NJ). Analyses were carried out by gas chromatography (HP 5890 Series II).

For the endotoxin removal experiments, vials of lyophilized *E. coli* O55:B5 endotoxin (Lonza Walkersville Inc., Walkersville, MD) were reconstituted and diluted. A uniform endotoxin film comprising about 2,200 endotoxin units (EU) was coated on the solid Ti surfaces. The contaminated surfaces were then treated with pure liquid CO_2 (27.6 MPa and 25 ºC) and liquid CO_2 + mixtures of water and surfactant in a pressure vessel for 2 hr. The molar water to surfactant ratio employed was selected from the data published by Liu et al. (2002) [16]. After treatment any residual endotoxin on the Ti surfaces was recovered by sonication. Samples were diluted (1:200) and assayed with the Limus Amebocyte Lysate (LAL) assay for endotoxin. Results from the processed disks were compared against untreated disks to determine endotoxin removal levels.

High level disinfection of poly (acrylic acid-co-acrylamide) potassium salt

Suspensions of *S. aureus* were first prepared and diluted to a concentration that ranged from 7.6×10^6 to 2.3×10^8 colony forming units per milliliter (cfu/mL). The model hydrogel (in dry powder) was then hydrated with the diluted bacteria suspension and transferred into a polyallomer centrifuge tube for CO_2 treatment (27.6 MPa and 40 ºC). Treatments were run from 10 minutes to 4 hours. Negative controls were evaluated by immersing untreated inoculated hydrogel in a laboratory temperature bath at 40 ºC for 4 hr. The amount of bacteria was quantified before and after treatment by a standard pulverization and plate counting procedure [17].

Sterilization of *Bacillus pumilus* spores

B. pumilus spore strips were exposed to sc CO_2 under controlled conditions (temperature, pressure, and time) in an ISCO SFX 2-10 two-cartridge fluid extractor (Lincoln, NE). Liquid additives (water, ethanol, and H_2O_2) at different concentrations were introduced into the

system. After the desired time, the cartridge was depressurized to atmospheric pressure. The cartridge containing the spore strips was immediately removed from the SFX 2-10 extractor. The degree of killing was quantified with a standard plate counting technique. The treated spore strips were transferred into a VWR Filtra Stomacher bag (LABPLAS Inc., Ste-Julie, Canada) containing 100 mL of sterile deionized water. Pulverization was then followed by placing the Filtra bag in a blender at 260 rpm. The contents were then heat-shocked at 80-85°C for 10 min in a circulator. Contents were quenched in an ice water bath (0-5°C) for 10 min. Each spore suspension was diluted serially by a factor of ten until a dilution of 10^{-5} was attained. Suspensions of each dilution were plated on petri dishes and incubated at 30-35°C. The number cfu on each petri dish was counted at both 24 and 48 hr. The log reduction of *B. pumilus* spores on a spore strip was then calculated.

RESULTS

Figure 1 gives the results from GC analysis in terms of oil removal by each cleaning process for the most significant peaks in the chromatograms occurred at retention times (RT) of 2.5, 4.1, 6.4, and 8.9 minutes. Based on Figure 1, higher or equal oil removal was achieved with sc CO_2 than with detergent for the main constituent species at the selected RT peaks. Efficiencies of 100% was attained for three of the evaluated RT (2.5, 4.1, and 8.9 min) and 99% for the fourth RT peak (6.4 min). These results indicate that sc CO_2 dissolved and removed the species observed for this oil.

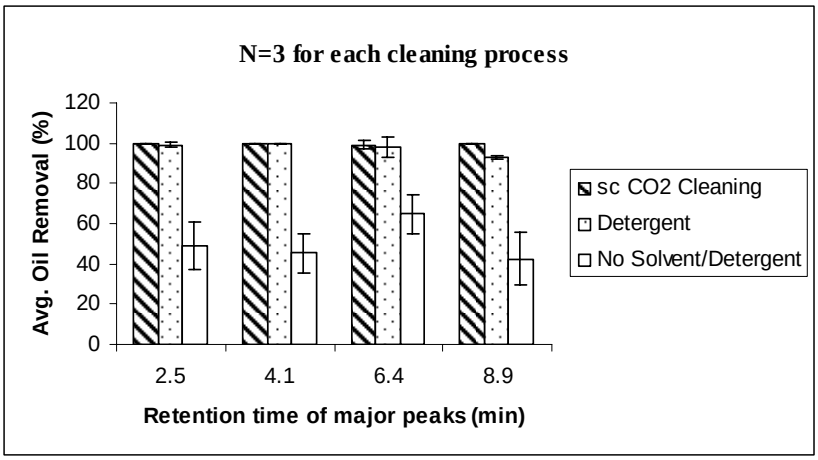

Figure 1: Average oil removal (%) for each cleaning process as quantified by GC analyses

The results for the endotoxin removal are presented in Figure 2. For experiments involving compressed CO_2 + surfactant and water, 100% endotoxin removal was attained. The probable mechanism is formation of water-in-CO_2 microemulsion; the water in the microemulsion will completely dissolve endotoxin in the fluid phase, facilitating its removal. For those experiments employing CO_2 with surfactant or water, 85% and 83% endotoxin removal was obtained, respectively. Pure CO_2 alone will not dissolve endotoxin; hence the low level of removal (17%) with pure CO_2 is probably due to agitation and physical dislodgment.

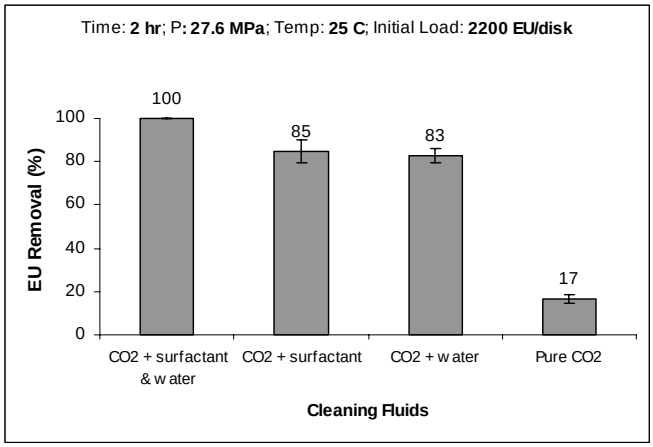

Figure 2: Endotoxin removal with liquid CO_2 and mixtures of water and surfactant

Figure 3 shows the effect of processing time on the average log-reduction of *S. aureus* in the model hydrogel after CO_2 treatment at 40°C and 27.6 MPa. The survival rate of *S. aureus* decreased roughly linearly with time until complete kill was achieved after 60 and 30 minutes of treatment. At times less than 30 minutes, incomplete kill is noted. The standard deviations become larger as the bacterial log-reduction increases. This occurs because the treatment time is in the proximity of the point where complete inactivation occurs. As the number of bacteria to count decreases, the experimental error increases.

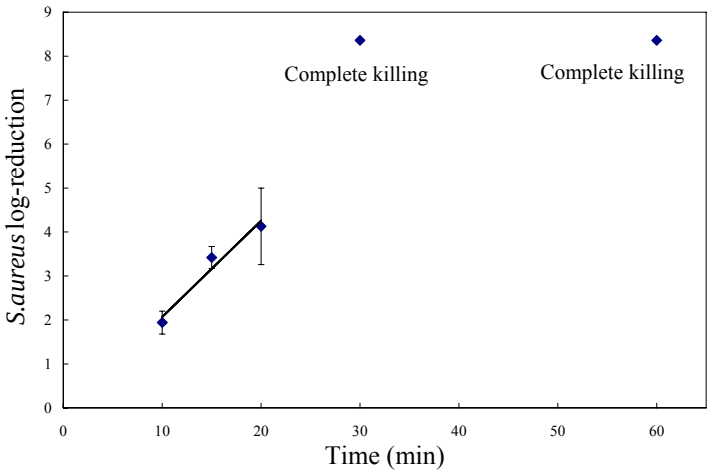

Figure 3: Log-reduction over time of *S. aureus* in poly(acrylic acid-co- acrylamide) after CO_2 treatment at 40 ºC and 27.6 MPa

In the sterilization experiments water was first employed as an additive. Because sc CO_2 with water did not achieve 6 log killing, H_2O_2 and ethanol were evaluated. In all subsequent experiments, 5µL of additive was injected with the CO_2. Table 1 shows the results for water, H_2O_2 (30%) and ethanol (70%). At 40 °C and 27.6 MPa, after a 4-hr treatment, ethanol gave only 0.27 log reduction. However, H_2O_2 was highly effective in killing dry spores. The killing rate varied from 4.45 (60°C, 27.5MPa, 2 hr) to 6.28 log reduction (60°C, 27.5MPa, 4 hr).

Table 1. Log-reduction achieved by sc CO_2 treatment with different additives

Additive	Temperature [ºC]	Pressure [MPa]	Duration [hr]	Log reduction
Water	50	27.6	4	0.58 ± 0.04
5 µL 70% etOH	40	27.6	4	0.27 ± 0.16
5 µL 30% H_2O_2	40	27.6	4	4.74 ± 0.64
5 µL 30% H_2O_2	50	27.6	4	4.60 ± 0.02
5 µL 30% H_2O_2	60	27.6	4	6.28 ± 0.46
5 µL 30% H_2O_2	60	27.6	2	4.45 ± 0.31

CONCLUSION

1. The lubricant oil removal study demonstrated that pure sc CO_2 at relatively low temperatures and moderate pressures can be used to remove oil from Ti surfaces. Moreover, it achieved equal or greater efficiency than washing with water and detergent.
2. The endotoxin removal study demonstrated that water-in-CO_2 microemulsion at room temperatures and moderate pressures (i.e. liquid region) can remove 100% of the endotoxin applied on Ti surfaces in 2 hr. High fractions of endotoxin were still removed when employing mixtures of liquid CO_2 with either water or surfactant, however safe endotoxin levels (≤ 20 EU/device as required for medical devices) were not achieved. Pure CO_2 did not remove significant amount of endotoxins because they are not soluble in CO_2.
3. After pure sc CO_2 treatment at 27.6 MPa and 40 ºC for 4 hr, complete kill of *S. aureus* embedded in poly(acrylic acid co-acrylamide) potassium salt hydrogel was achieved. Therefore, pure CO_2 treatment is sufficient to achieve a high level of inactivation at the reported experimental conditions.
4. A 6 log reduction of *B. pumilus* was achieved when adding trace levels of H_2O_2 to sc CO_2 (27.6 MPa and 60 ºC) for a 4 hr treatment.

The successful removal of bio-contaminants such as oils and endotoxins from Ti surfaces with compressed CO_2 indicates a promising alternative technology for the final cleaning of biomaterials and reused medical devices. This technology is particularly attractive for this application for many reasons including waste minimization and hazardous solvent elimination. The novel CO_2 process completely kills bacteria and disinfected the model hydrogel. Successful development of this technology would remove a major barrier to commercialization of implantable biopolymers, especially those being developed for cell-based tissue engineering.

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