Published in final edited form as:

J Supercrit Fluids. 2011 January 1; 55(3): 1052–1058. doi:10.1016/j.supflu.2010.09.010.

Removing Endotoxin from Metallic Biomaterials with Compressed Carbon Dioxide-Based Mixtures

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Abstract

Bacterial endotoxins have strong affinity for metallic biomaterials because of surface energy effects. Conventional depyrogenation methods may not eradicate endotoxins and may compromise biological properties and functionality of metallic instruments and implants. We evaluated the solubilization and removal of *E. coli* endotoxin from smooth and porous titanium (Ti) surfaces and stainless steel lumens using compressed CO₂-based mixtures having water and/or surfactant Ls-54. The CO₂/water/Ls-54 ternary mixture in the liquid CO₂ region (25 °C and 27.6 MPa) with strong mixing removed endotoxin below detection levels. This suggests that the ternary mixture penetrates and dissolves endotoxins from all the tested substrates. The successful removal of endotoxins from metallic biomaterials with compressed CO₂ is a promising cleaning technology for biomaterials and reusable medical devices.

Keywords

endotoxin; carbon dioxide; microemulsion; cleaning; biomaterial; surfactant

1. Introduction

Carbon dioxide (CO₂) in the liquid and supercritical (SC) state has been proposed as a useful replacement for toxic organic solvents in cleaning applications [1–4]. A key impetus is that CO₂ efficiently penetrates complex matrices because of the liquid-like densities and gas-like viscosities. However, the use of compressed CO₂ as a cleaning agent is limited because of its low solvation capacity in dissolving high molecular weight and hydrophilic substances, which are a main target in biomedical cleaning applications. This limitation can be overcome by adding small amounts of suitable surfactants to support water-in-CO₂ microemulsions. The aim is to disperse water in the CO₂ continuous phase, allowing solubilization of large and polar compounds. Early efforts toward this goal led to development of several fluorinated surfactants [5–10].

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Because of the high costs and potential toxicity of fluorinated surfactants, several investigators have focused on finding hydrocarbon based surfactants. Liu *et al.* [11] reported that the commercial surfactant Dehypon® Ls-54 is soluble in supercritical CO₂ and enables formation of water-in-CO₂ microemulsions. Dehypon® Ls-54 is an ethoxylated alcohol that contains only C, H, and O and does not incur the environmental concerns of fluorinated surfactants. Its chemical structure is shown below

Hence, this article examines the capability of CO₂ + Ls-54/water mixtures to solubilize bacterial endotoxins, a complex pathogenic substance, from metallic biomaterials. The solubility of Ls-54 in SC CO₂ was previously measured [11] and was reported to be 0.05 M at 35 °C and 22 MPa. A variety of molar water-to-surfactant ratios (W₀) forming a microemulsion phase at different pressures and temperatures were also reported [11]. We utilized W₀ = 12 as the most appropriate for the temperature and pressure range of this work.

Endotoxins are potent pyrogens that have strong affinity for metals because of surface energy effects [12–13]. Conventional depyrogenation techniques include dry heat and chemical inactivation. However, these can be problematic because they can degrade the material and alter its properties [14]. Other approaches include rinsing and washing with non-pyrogenic solvents, which can lead to unacceptable levels of residual endotoxin [13]. Moreover, water-based processes are hampered by the high surface tension of water, which does not allow penetrability into porous devices and may not be sufficient for certain applications.

This study first establishes the viability of the Ls-54/water/CO₂ mixtures in removing *Escherichia coli* endotoxins from smooth titanium (Ti) surfaces. Subsequent work focuses on substrates with complex morphologies found in medical devices, namely, narrow stainless steel lumens (significant for devices such as catheters and gastrointestinal endoscopes) and porous-coated Ti surfaces (commonly found in orthopaedic implants). Such geometries present flow and mass transfer limitations.

2. Materials and methods

2.1 Chemicals and bio-contaminant

Dehypon® Ls-54 surfactant was donated by Cognis Corporation (Ambler, PA). Bone-dry grade CO₂ (National Welders Supply Co., Durham, NC) with 99.8% purity was used as the main cleaning solvent. *E. coli* O55:B5 endotoxin (Lonza Walkersville Inc., Walkersville, MD) was the bio-contaminant. Endotoxin-free water (HyClone Laboratories Inc., Logan, UT) was used at all times. The Limulus Amebocyte Lysate (LAL) Kinetic-QCL assay kit (Lonza Walkersville Inc.) was employed to determine endotoxin levels.

2.2 Substrates

Commercially pure Ti disks with smooth surfaces (12 mm in diameter and 2.5 mm in thickness) were provided by Dr. Yuehuei An of the Medical University of South Carolina. Stainless steel tubing (Valco Instruments Co. Inc., Houston TX) of 3.175 mm (1/8 in) OD and 2.159 mm (0.085 in) ID were used to simulate lumens. Two lengths were evaluated; 102 mm (4 in) and 610 mm (24 in). Porous-coated coupons measuring 6 mm in diameter and 12 mm in length were donated by Smith and Nephew, Inc., Memphis, TN. The porous layer is 1 mm thick with an approximated total porosity of 35% and a pore size of 150 µm. The porous

surface is made from commercially pure Ti, which represents porous-coated surfaces used on acetabular shells, femoral stems, and knees.

2.3 Depyrogenation

Before each experiment, all substrates, pipettes, and other glassware were depyrogenated in a dry heat oven (Fisher Scientific Isotemp Oven, model 725F) at 250 °C for at least 30 minutes [15–16].

2.4 Endotoxin reconstitution and stock solution preparation

Vials of lyophilized *E. coli* endotoxin (2.5 mg/vial; nominal 7.5×10^6 EU) were reconstituted as specified by the supplier and diluted with endotoxin-free water to obtain multiple stock solution concentrations.

2.5 Endotoxin solubilization and removal from simple geometries

Well-polished and passivated (according to ASTM Standard F86-76) Ti disks were first coated with endotoxins by applying 200 μ L from a stock solution of approximately 12,000 EU/mL. The disks were dried in a biohood at room temperature, leaving a film of approximately 2,000 – 2,500 EU per disk. The coated Ti disks were then processed with compressed CO₂-based mixtures. The amounts of water and Ls-54 added into the system were calculated based on the size of the pressure vessel and the reported solubility for Ls-54 in CO₂ [11] and the selected W_0 .

The processing was carried out in a 1 L pressure vessel (FC series, Pressure Products Industries) for a temperature and pressure range of 5 to 40 °C and 13.8 to 27.6 MPa, respectively, with no stirring/mixing to the fluids. A schematic of the apparatus is shown in Figure 1. A total of four disks (3 coated; 1 endotoxin-free) were secured in a stainless steel plate, which is attached to a shaft inside the pressure vessel. After a specified time, the vessel is slowly depressurized. The presumed mechanism of endotoxin removal is by solubilization in the CO₂ mixture. The function of the endotoxin-free disk (control) is to ascertain whether the solubilized endotoxin might re-deposit during the cleaning treatment or during the depressurization step.

Further evaluations were conducted in a phase equilibrium monitor (PEM) vessel (SPM 20, Thar Technologies Inc., Pittsburgh PA) to study the effect on the endotoxin removal when providing stirring (mixing) to the cleaning fluids. This is a pressure vessel of 23 mL in volume, which has a motor-driven stirrer that allows high stirring rates (up to 3,800 rpm). A schematic of the apparatus is given in Figure 2. Due to the volume limitation in the PEM system, only one disk could be treated at a time.

Endotoxin recovery was achieved by sonication in an ultrasonic cleaner (model 250D, VWR). After treatment, all Ti disks were placed individually in a 40 mL depyrogenated glass bottle with 20 mL of endotoxin-free water and sonicated for 10 minutes. To provide a baseline against which to evaluate CO₂ cleaning, negative controls (untreated, coated Ti disks) were also sonicated in endotoxin-free water for 10 minutes. After sonication, the eluates were diluted (1:200) and analyzed with the LAL assay.

2.6 Inoculation and processing of lumens and porous Ti coupons

A stock solution of approximately 30,000 EU/mL was drawn through the length of the depyrogenated stainless steel lumens with the use of a syringe. The lumens were then capped on the bottom and placed vertically in a vacuum oven (model 1450M, VWR) for approximately 17 h at 70 °C and 50.5 kPa to evaporate the water, leaving the endotoxin coated on the interior.

The open porosity (i.e. porosity accessible to water intrusion) in the porous Ti coupons was first evaluated experimentally by the intrusion porosimetry method [17]. Then inoculation was carried out by submerging the porous-coated coupons in an endotoxin stock solution of known concentration contained in a pressure vessel. The vessel was sealed and pressurized to 0.55 MPa (80 psi) for 2 minutes to force the stock solution into the porous layer. Coupons were then removed and placed in a dry heat oven (Fisher Scientific Isotemp Oven, model 725F) at 60 °C for 2 h to evaporate the water within the pores.

Both lumens and coupons were weighed in an analytical balance (model XS105 DualRange, Mettler-Toledo Inc., Columbus OH) to confirm that all water was completely evaporated. Endotoxin-contaminated lumens and coupons were processed separately for 2 h in the same 1 L pressure vessel configuration shown in Figure 1. Bulk agitation was provided by a flat-blade impeller rotating at 1900 rpm. The conditions were 27.6 MPa and 25 °C. Endotoxin recovery was carried out by placing the substrates separately in depyrogenated glass containers with endotoxin-free water and sonicating for 10 minutes. Immediately following the endotoxin recovery procedure, the eluates were diluted (1:200) and assayed.

2.7 Endotoxin detection assay

Endotoxin levels were assayed using the chromogenic LAL Kinetic-QCL assay, which has a sensitivity range of 0.005 – 50 EU/mL. Diluted samples from the recovery process were placed in a multi-detection microplate reader (model Synergy HT, Bio-Tek Instruments, Inc.) and incubated for 10 minutes at 37 °C. After the initial incubation, the LAL reagent was added and the samples were automatically monitored photometrically over time at a 405 nm wavelength. The concentration of endotoxin in a given sample was then calculated from the reaction time by comparison to the reaction time of solutions containing known amounts of endotoxin standard.

3. Results and Discussion

3.1 Treatment of smooth Ti disks in the 1-L pressure vessel

Table I summarizes nine different treatments (temperature, pressure, duration and endotoxin loadings) for the disks that were processed in the 1 L pressure vessel. Figure 3 shows initial and residual endotoxin levels after treatments 1 – 4. The endotoxin removal percentage for each treatment is also displayed. Neither pure SC CO₂ nor liquid CO₂ (treatments 1 and 3) removed a significant fraction of endotoxin from the Ti surfaces. This is as expected, because CO₂ alone has insufficient solvent strength to dissolve the large endotoxin biomolecule. However, adding both Ls-54 and water together enhanced the removal to 93% and 81% in the liquid and SC CO₂ regions (treatments 2 and 4), respectively. We infer that a water-in-CO₂ microemulsion system forms in both the liquid and SC phases.

The precise microstructure of the Ls-54 + water + CO₂ mixture below the critical temperature has yet to be established. One possibility is that a microemulsion phase forms, similar to that reported by Liu *et al.* [11]. At a given pressure, Ls-54 has a higher solubility in CO₂ at lower temperatures according to Liu *et al.* [11], and this might support microemulsion formation in liquid CO₂. Without direct spectroscopic observation of a microemulsion, and considering the difficulty in forming and stabilizing such phases (as reported by Adkins *et al.* [18]), other phase behavior may be more likely. The surfactant may merely act to reduce surface tension between water, CO₂, and the substrate, facilitating endotoxin removal. The formation of a reverse (CO₂ in water) phase cannot be ruled out at this time.

Liquid and SC CO₂ mixtures decreased the endotoxin levels in the disks to 164 and 498 EU/disk, respectively, from an initial loading of approximately 2,500 EU/disk (Figure 3). It is

desirable to reduce endotoxin to less than 20 EU/disk (safe residual endotoxin level for medical devices according to the United States Pharmacopeia and National Formulary, USP27-NF22). Additional experiments were conducted in the liquid CO₂ region prolonging the duration of the treatment. However, the residual endotoxin levels were 78.67 ± 89 EU/disk after 12 h of treatment. Safe residual endotoxin levels as stated by the USP might be feasible with a two-stage process. Hence, experiments with lower initial endotoxin loading (treatment 5 in Table I) were conducted in the 1 L pressure vessel with the ternary mixtures in the liquid CO₂ phase. This treatment resulted in an average endotoxin level of 12 ± 21 EU/disk for an average removal of 97%. This is below the established USP requirements for medical devices and suggests that a two-stage process, using a liquid CO₂ mixture without significant agitation (stirring) might yield to a 99.5% removal of endotoxin for surfaces initially coated with 2,500 EU.

Shorter duration and lower pressure were also evaluated (treatments 8 and 9 in Table I). The average endotoxin level after 2 h treatment at 27.6 MPa and 5 °C was 1296 ± 189 EU/disk, corresponding to $59 \pm 2.4\%$ removal. For the 4 h treatment at 13.8 MPa and 5 °C the average endotoxin removal was $57 \pm 8\%$. Both of these treatments were less effective than the 4 h treatment at 27.6 MPa. According to Liu *et al.* [11] the solubility of Ls-54 in CO₂ decreases with pressure. Without vigorous mixing in the 1 L pressure vessel, there is a less of energy either to promote mass transfer or (possibly) to form a microemulsion, thus longer time is needed for the system to reach equilibrium and achieve complete endotoxin removal. Therefore, it is expected that decreasing the duration or pressure of the treatment would reduce the efficiency of endotoxin removal.

As described in Section 2.5, we considered the possibility of endotoxin re-deposition during the cleaning process. A non-contaminated, clean disk (control) was processed and analyzed after every trial. There was no evidence of endotoxin on the control disk. Therefore, we assumed that the dissolved endotoxin was flushed out of the system after depressurization.

Endotoxin removed as shown in Figure 3 was obtained in the 1 L pressure vessel, with no bulk agitation. Subsequent disk cleaning treatments in the PEM vessel allowed vigorous bulk agitation. These treatments were done at the conditions that gave the highest endotoxin removal in the 1 L pressure vessel. Thus, all treatments were conducted in the liquid CO₂ region (25 °C; 27.6 MPa). Treatments were done with pure liquid CO₂, CO₂ + Ls-54, CO₂ + water, and CO₂ + Ls-54 + water.

Figure 4 shows the residual endotoxin on the Ti disks after cleaning in the PEM vessel. Also, it is shown the residual endotoxin on a surface washed with water + Ls-54 (positive control). As seen in Figure 4, pure CO₂ removed 17% of the endotoxin coated to each disk surface. The small level of removal is probably due to higher agitation and physical dislodgment, because endotoxin does not dissolve in CO₂. When Ls-54 or water were added individually to CO₂, the endotoxin removal was 85% and 83%, respectively. This indicates that these additives individually impart some solubilization of the endotoxin. However, no residual endotoxin was detected on the Ti surfaces cleaned with the ternary Ls-54 + water + CO₂ mixture. Thus, vigorous agitation facilitates higher solubilization of the endotoxin, making possible its complete removal in 2 h.

Adding just Ls-54 to CO₂ causes the disassociation of endotoxin aggregates due to the surfactant effect, as shown in the literature [19–24]. In this study we achieved 85% endotoxin removal when processing the Ti disks with liquid CO₂ + Ls-54. We infer that in this scenario the surfactant weakened the endotoxin aggregation, facilitating higher removal by CO₂. Another possibility is that an inverse micelle could have formed between CO₂ and

the surfactant, allowing the endotoxin removal. Further studies will address these hypotheses.

Reduced endotoxin levels (83%) were also appreciable after processing the Ti disks with liquid CO₂ + water (83% reduction). Despite the fact that CO₂ has a noticeable solubility in water [25–26] and dissociates to form carbonic acid, inactivation of the endotoxin molecule due to acid hydrolysis is not expected. Furthermore, carbonic acid is also a weak acid [27–28], so it is unlikely that endotoxin degradation occurs. Additional experimental work was conducted to examine endotoxin levels after exposure to carbonic acid solutions (~ pH = 3.4). After 2 h of exposure, the endotoxin stock solutions were assayed and no endotoxin degradation was measured from the LAL assay. Consequently, the endotoxin attenuation after CO₂-water treatment was most likely due to endotoxin removal. It is believed that water acted as a co-solvent, increasing important bulk properties for solvation in CO₂ such as the dielectric constant and polarity [29–31], thus enhancing endotoxin removal. For instance, the dielectric constant for the mixture of CO₂ and water will be 2.46, whereas for pure CO₂ at the same conditions is 1.59.

The beneficial effects of water as a co-solvent have been previously observed and reported for the extraction of bioactive compounds [32] and lipopolysaccharides (LPS) [33] in SC CO₂ systems. In addition, LPS molecules contain long carbohydrate chains that favor its solubility in water. As shown in Figure 5, the amphiphilic LPS molecule consists of a hydrophobic region denoted by a lipid chain (Lipid A), and a polysaccharide section (O-antigen and Core Region) that maintains the hydrophilic domain of the molecule. We speculate that the hydrophilic group in the endotoxin (which is larger than the hydrophobic region) dissolves in the mixture of liquid CO₂ + water, thus facilitating its removal.

Because endotoxin exhibits high solubility in water, an assessment was carried out for comparison by cleaning a contaminated surface with water as the main solvent (i.e. no CO₂). This scenario mimics traditional aqueous-based cleaning processes. Three cleaning trials (n=3) were conducted by washing endotoxin-coated Ti disks in DI water + surfactant Ls-54 for 2 h at the same temperature (25 °C) and stirring (1900 rpm) employed in the PEM vessel. Same surfactant-to-solvent ratio as that employed for the CO₂ cleaning was maintained. As seen in Figure 4, the average endotoxin removal was 92%. Considering that the processed substrates are flat, well-polished metal surfaces and that endotoxins are soluble in water, the high endotoxin removal is not surprising. Twohy and Duran [35] showed that high fractions of endotoxin could be removed from devices by a combination of rinsing, sonication and vortexing. In addition, surfactants lower the surface tension of aqueous cleaning fluids, which increases the wetting of the substrate, and facilitates endotoxin removal. However, a considerable amount of residual endotoxin (~178 EU) still remained on the disk surface. This indicates, as expected, that the water-based solution was inadequate to dissolve and remove all the endotoxin. A t-test for a 95% confidence level was used to compare the water-based process against the liquid CO₂ ternary mixture. The difference in endotoxin removal was statistically significant, meaning that liquid CO₂ ternary mixture treatment is superior.

3.2 Treatment of Stainless Steel Lumens

In this part of the study, stainless steel lumens of two different lengths (102 and 610 mm) were contaminated with endotoxin and then treated with the CO₂-based mixtures. The 102 mm lumens were cleaned first to establish the effectiveness of the CO₂-based cleaning. Subsequent treatments were conducted with 610 mm lumens, a length which is more representative of actual medical devices such as rigid laparoscope in endoscopes.

As with the Ti disks, the maximum recoverable endotoxin from each lumen was defined as the endotoxin recovered by sonication of negative controls. Endotoxin levels from the treated lumen were then compared to the average negative control to determine the percentage endotoxin removal. For the 102 mm lumens, three individual specimens were used throughout the study. These three specimens were inoculated and processed simultaneously. On average 4274 ± 682 , 4154 ± 398 , and 4345 ± 546 EU ($n=3$) were recovered from untreated lumens 1, 2, and 3. Because of space limitations in the 1 L pressure vessel, only one 610 mm lumen could be processed at a time. The average endotoxin recovered from the negative control in the 610 mm lumen was $26,900 \pm 4800$ EU ($n=3$). It must be pointed out that the endotoxin level in the 610 mm lumen is over 200 times greater than the highest endotoxin level found in reusable angiographic catheters (450 – 1100 mm in length) as reported by Kundsinn and Walter [36]. This high endotoxin loading, along with the fact that stainless steel supports endotoxin adherence, presents a strong cleaning challenge.

No residual endotoxin was detected for lumens cleaned with the liquid CO₂ ternary mixture process (i.e. CO₂ + water + Ls-54). However, as expected, pure liquid CO₂ did not remove a significant fraction of endotoxin. On average, after pure CO₂ treatment the remaining endotoxin in the 102 mm lumens was 3585 ± 61 , 3222 ± 99 , and 3678 ± 84 EU.

Figure 6 shows the residual endotoxin levels on the long lumens (602 mm) after being cleaned with compressed CO₂ or water + Ls-54 (no CO₂). No residual endotoxin is detected on the lumen cleaned with liquid CO₂ + water + Ls-54. These results indicate that there was facile penetration of the additives with the CO₂ solvent into the long lumen. However, only 62% of the endotoxin was removed from the lumen cleaned with the mixture of Ls-54 + water. Note that removal of endotoxin was not as high as for the flat Ti surfaces when washed by the Ls-54 + water approach. This indicates, as expected, that the water-based solvent did not effectively penetrate the narrow lumen to dissolve and remove the endotoxin. Pure liquid CO₂ did not remove a significant fraction of endotoxin either. On average, after pure CO₂ treatment the remaining residual endotoxin was $24,250 \pm 760$ EU.

3.3 Treatment of porous Ti coupons

The initial mass of endotoxin inoculated on each porous coupon was calculated from the open void volume and the stock solution concentration. The maximum recoverable endotoxin was defined as the endotoxin recovered from an untreated, inoculated coupon upon sonication in endotoxin-free water. These were the negative controls. On average 81% of the calculated endotoxin was recovered from the untreated porous layer after 10 minutes of sonication with endotoxin-free water.

Figure 7 summarizes the endotoxin levels present in the porous Ti coupons before and after treatment. After 2 h at 27.6 MPa and 25 °C, no endotoxin was detected in the coupons when processed with CO₂ + Ls-54 + water. This indicates that the ternary mixture completely penetrated the porous layer and solubilized all the endotoxin. It is evident that the pore size (150 µm) did not pose a mass transfer barrier. The possible removal mechanism is the solvation of the endotoxin by the liquid CO₂ ternary mixture or possibly by the aqueous phase in the interior of the microemulsion (if a microemulsion was present). As expected, pure liquid CO₂ did not remove a high percentage of endotoxin. The residual endotoxin after processing with pure CO₂ (under the same conditions) averaged 454 ± 26 EU per coupon, or 12.5 ± 10 % removal.

As in the previous trials, the endotoxin removal was quantified after cleaning with a mixture of water and Ls-54. Three trials ($n=3$) were carried out. On average, 60% of endotoxin was removed by the water/Ls-54 mixture. Because surfactants lower the surface tension of

aqueous cleaning fluids, which increases the penetration and wetting, some fraction of the endotoxin will be removed. Hence, endotoxin removal may be facilitated by adding surfactant to the water. However, despite the 60% endotoxin removal, residual endotoxin (~236 EU) still remained within the porous layer. This indicates, as expected, that the water-based solution did not effectively penetrate and dissolve all the endotoxin. Since DI water has relatively high surface tension, the wetting ability is low [37] leading to poor penetrability into the porous layer. Consequently, complete removal of the endotoxin by the water/Ls-54 mixture will be difficult.

3.4 Endotoxin removal with flow and mass transfer restrictions

To further evaluate endotoxin removal, additional experiments were carried out wherein flow and mass transfer restrictions were imposed. As shown in Figure 8, a physical barrier consisting of a 5 μm porous frit was placed in the PEM vessel to restrict the circulation of the cleaning fluids through the contaminated surfaces (Ti disks).

Configuration 8a simulates a cleaning process with mass transfer through a porous barrier. In configuration 8b agitation is provided directly to the CO_2 above the frit, but not to the water and surfactant which are located below the frit. The main difference between configurations is that 8a allows stirring of all cleaning fluids (CO_2 plus additives) while 8b allows stirring only of CO_2 .

Figure 9 shows the endotoxin removal in the PEM vessel system under configurations 8a and 8b. The initial load of endotoxin was 2,200 EU/disk. Although strong stirring was provided, endotoxin removal was low (16% and 37%, respectively) because of the mass transfer limitations. For configuration 8a, the ternary mixture must migrate through the porous frit to dissolve and remove the endotoxin from the disk surface. Because of this strong mass transfer limitation, additional time is required to achieve complete endotoxin removal. In configuration 8b, the liquid CO_2 is initially separated from the Ls-54 and water by the porous frit. Agitation is applied to the contaminated surface where liquid CO_2 is introduced. Because the contaminated surface is directly exposed to the rotating impeller, it can be inferred that some of the endotoxin removal is due to the high agitation and physical dislodgment. This configuration (8b) might prevent water from reaching the Ti surface having both diffusion and microemulsion formation limitations (in the advent of a microemulsion phase).

4. Conclusions

The present study demonstrated that the CO_2 -based mixtures with water and surfactant Ls-54 can solubilize and dissolve *E. coli* endotoxins. Also, it was demonstrated that in the liquid CO_2 region, the ternary mixture works better than either binary with CO_2 . For conditions with strong mixing, the ternary liquid mixture (25 $^{\circ}\text{C}$ and 27.6 MPa) removed all detectable endotoxin applied on all tested substrates (smooth and porous Ti surfaces and stainless steel lumens) significant for medical applications.

Under poorly mixed conditions, longer periods of time (> 4 h) were required to attain ≤ 20 EU in the Ti disks. The study also indicates that endotoxin removal will be dependent on diffusion to the contaminated surface. Significant fractions of endotoxin (83 to 85%) were also removed from the Ti disks when employing binary mixtures of liquid CO_2 with either water or Ls-54. Pure CO_2 , whether in the liquid or SC region, did not remove significant amount of endotoxins from the Ti disks and lumens because they are not soluble in CO_2 .

The ability of the CO_2 /water/surfactant mixture to remove endotoxin is very promising and may represent a realistic alternative for situations involving difficult-to-clean, porous and

narrow-lumen medical devices. As the design of medical devices becomes more complex, new techniques to assure proper endotoxin removal must be developed as well. Compressed CO₂ at room temperature and relatively low pressure with a small fraction of Ls-54 surfactant and water completely removed endotoxins from stainless steel lumens and porous-coated substrates. The use of CO₂ is favorable because CO₂ is inexpensive, non-toxic, non-flammable, and is readily available from industrial sources.

Acknowledgments

The authors acknowledge the National Institutes of Health (NIH) for partially supporting this work under a Bioengineering Research Partnership grant (R01EB55201). Financial assistance for Pedro J. Tarafa was provided by the Alfred P. Sloan Foundation and the South East Alliance for Graduate Education and the Professoriate (SEAGEP). The helpful comments of Professor James Blanchette and the reviewers are greatly appreciated.

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Figure 1. Schematic of the 1 L pressure vessel apparatus. (1) CO₂ gas cylinder; (2) pump; (3) water/coolant bath; (4) pressure vessel; (5) stainless steel plate attached to the shaft; (6) cooling coil; (7) heating jacket; (8) pressure indicators.

Figure 2.
Schematic of the PEM vessel apparatus.

Figure 3. Initial and residual endotoxin levels (Mean $\pm$ SD) after processing the Ti disks for 4h with pure CO₂ and CO₂-based mixtures in the 1 L pressure vessel.

Figure 4. Residual endotoxin (Mean $\pm$ SD) on Ti disks after processing with liquid CO₂ and mixtures of water and Ls-54 with stirring/mixing.

Figure 5.
Chemical structure of *E. coli* endotoxin (source: Ohno and Morrison[34]).

Figure 6.
Residual endotoxin after processing long stainless steel lumen (610 mm).

Figure 7. Residual *E. coli* endotoxin on porous Ti coupons after processing with pure liquid CO₂, water + Ls-54, and liquid CO₂ + Ls-54 + water.

Figure 8.
Schematic of the PEM vessel with flow and mass transfer restrictions.

Figure 9. Percent of endotoxin removal (Mean $\pm$ SD) from Ti disks using liquid CO₂ + Ls-54 and water for configurations as described by Figure 8 in the PEM system.

Table I
Experimental treatments conducted in the 1 L pressure vessel for removing endotoxin from Ti disks.

Treatment	Cleaning Fluid (s)	T (C)	P (MPa)	Duration (h)	Initial loading (EU/disk)
1	Supercritical (SC) CO ₂	40	27.6	4	2900
2	SC CO ₂ + Ls-54 & water	40	27.6	4	2628
3	liquid CO ₂	5	27.6	4	2502 ± 71
4	liquid CO ₂ + Ls-54 & water	5	27.6	4	2348 ± 82
5	liquid CO ₂ + Ls-54 & water	5	27.6	4	440 ± 32
6	liquid CO ₂ + Ls-54	5	27.6	4	2970 ± 457
7	liquid CO ₂ + water	5	27.6	4	2618 ± 265
8	liquid CO ₂ + Ls-54 & water	5	13.8	4	2169 ± 810
9	liquid CO ₂ + Ls-54 & water	5	27.6	2	3145 ± 438