

Supercritical Carbon Dioxide-Peracetic Acid Treatment Inactivates Low-Level Bacterial Inocula in Collagen Analogue-Phosphorylcholine Hydrogels while Preserving Functional Properties

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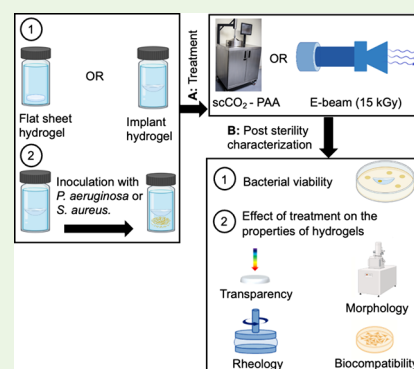
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ABSTRACT: Terminal sterilization of hydrogels is challenging because their high-water-content, chemical structures, and cell-friendly properties are often altered by conventional sterilization methods. Hydrogel implants comprising collagen-like peptide (CLP), polyethylene glycol (PEG), and poly (2-methacryloyloxyethyl phosphorylcholine) (MPC) showed promise in promoting corneal regeneration in preclinical animal studies. However, before these CLP-PEG-MPC implants can be used clinically, they must be sterilized effectively to ensure patient safety. Here, we evaluated supercritical carbon dioxide (scCO₂) with peracetic acid (PAA) as a potential sterilization technique for hydrogels. CLP-PEG-MPC was treated with scCO₂ containing PAA and compared against 15 kGy of electron beam irradiation (E-beam) and chloroform (CF) sterilization. Several samples were spiked with 200 colony-forming units (CFU) of either *Pseudomonas aeruginosa* or *Staphylococcus aureus* prior to sterilization to compare the bactericidal efficacy of the three sterilization methods. Only scCO₂-PAA effectively inactivated all the inoculated bioburden. Non-spiked hydrogels were also sterilized and then compared for complement activation, changes to their physical and mechanical properties, and biocompatibility. Optical clarity was unchanged regardless of sterilization mode. scCO₂-PAA did not alter the mechanical properties of the hydrogels but resulted in a modest lowering of corneal epithelial cell proliferation, while E-beamed hydrogels showed reduced mechanical strength. In conclusion, newer scCO₂-PAA terminal sterilization preserves the functional properties of soft hydrogels, such as CLP-PEG-MPC, including transparency, which is needed for corneal implants.

KEYWORDS: supercritical carbon dioxide-peracetic acid, electron beam irradiation, chloroform sterilization, hydrogel implants, cornea



1. INTRODUCTION

Hydrogels make excellent implants for repairing or enhancing various tissues and organs, as their high-water content allows them to mimic the properties of the body's soft tissues.¹ However, the same high-water content and complex biologically mimicking structures in hydrogels render them difficult to sterilize using traditional methods such as autoclaving, chemical treatment (ethylene oxide), and electromagnetic radiation (gamma or ultraviolet).² Hydrogel-based artificial corneal implants comprising EDC/NHS cross-linked recombinant human collagen type III (RHCI) and interpenetrating networks of RHCI and the anti-inflammatory polymer poly(2-methacryloyloxyethyl phosphorylcholine) (MPC)^{5,6} have been successfully tested in humans. Collagen was used to fabricate the corneal implants, as it is the primary structural component of the native cornea.⁷ The implants were prepared by aseptic processing, and sterility was maintained by adding 1% chloroform (CF) into each vial, which was then sealed aseptically.³⁻⁶ The resulting implants were sterile with endotoxin levels within

the accepted limits as tested by a certified lab before use in patients. However, this storage solution required an extensive washing procedure to remove the chloroform, followed by additional washing in antibiotics to ensure sterility before surgery. Therefore, beyond the first-in-human feasibility trials in a small number of patients, future clinical studies and eventual clinical application will require sterilization methods that allow the surgeon to open a vial and use the implant directly.

RHCI-MPC implants can be terminally sterilized using electron beam irradiation and can retain their preregeneration properties, but their poststerilization physical and functional

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properties are dependent on the radiation dose received.⁸ Recombinant RHCIII, like native type III collagen, is a large and relatively chemically inert macromolecule and difficult to process and handle.⁹ For increased manipulability, we switched to a fully synthetic collagen analogue comprising short synthetic collagen mimetic peptides (CMPs) or collagen-like peptides (CLPs) conjugated to polyethylene glycol (PEG). CLP-PEG implants containing MPC, like RHCIII-MPC, promoted regeneration of corneal tissues when tested preclinically in the corneas of mini-pigs^{10,11} and recently in rabbits.¹² As with RHCIII-MPC implants, a terminal sterilization method is also needed for CLP-PEG-MPC implants to ensure sterility while preserving functionality since graft contamination has been associated with severe infections in recipients.^{13,14}

Supercritical carbon dioxide (scCO₂) has been proposed for sterilization of heat- and radiation-sensitive biomaterials, including biologically derived tissues such as bone allografts and decellularized tissues such as aortas and corneas.^{15–18} It is nonflammable and nontoxic.¹⁹ Under supercritical conditions (low pressure and temperature), CO₂ exists as a liquid with low viscosity and zero surface tension, allowing it to penetrate complex structures and porous materials effectively.^{18,20} Used in combination with peracetic acid (PAA), CO₂ effectively permeates the cell walls of bacteria and other microorganisms, bacterial endospores, and viruses to inactivate them.^{15,21}

Here, we examined the sterilization efficacy of scCO₂-PAA and compared it with that of electron beam (E-beam) irradiation and CF sterilization for inactivating bacteria while preserving the functional properties of the CLP-PEG-MPC hydrogels. To date, scCO₂-PAA has not been evaluated for its capacity to maintain the function of synthetic biomimetic hydrogels, including transparency critical for corneal implants.

2. MATERIALS AND METHODS

2.1. Preparation of CLP-PEG-MPC Hydrogels

Briefly, a custom designed CLP with the sequence (CG(PKG)₄(POG)₄(DOG)₄)^{10,11} (Genscript, Piscataway, NJ, USA) was conjugated to a 40 kDa 8-arm poly (ethylene glycol) maleimide with a tripentaerythritol core (Sinopeg Biotech Co. Ltd., Xiamen, China) at pH 4.5. The resulting CLP-PEG conjugate was filtered and dialyzed using a 1–2 kDa cutoff membrane to remove unreacted CLP and then lyophilized. Hydrogels were prepared at a CLP-PEG to MPC ratio of 2:1 (w/w). To prepare the hydrogels, the lyophilized CLP-PEG was dissolved in 0.625 M 2-(*N*-morpholino) ethanesulfonic acid (MES; Sigma-Aldrich, Schnelldorf, Germany) buffer. The solution was heated at 65 °C with constant stirring at 1000 rpm using a thermomixer. A brief centrifugation (1 min at 10,000 rpm) was performed to facilitate the immersion of the solid material. The solution was then returned to the thermomixer and maintained at 65 °C until fully dissolved. MPC (Paramount Fine Chemicals, Dalian, China) dissolved in 0.625 M MES was added to the CLP-PEG solution, followed by polyethylene glycol diacrylate (PEGDA) and *N,N,N',N'*-tetramethylethylenediamine (TEMED) (Sigma-Aldrich, St. Louis, MO, USA). Ammonium persulfate (APS) and 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) (Sigma-Aldrich USA) were used as cross-linking agents. The final weight ratios were CLP-PEG-MPC at 2:1, with MPC:PEGDA at 3:1. PEGDA cross-linked the MPC network through radical polymerization initiated by APS and TEMED. Corneal-shaped and flat sheet hydrogels were molded and consequently stored for 6 months to evaluate their mechanical and optical properties.

Hydrogels were fabricated in an ISO7 cleanroom. Corneal-shaped implants fabricated for sterility testing were first tested for sterility and endotoxin levels to establish a baseline before starting the

sterilization experiments. The sterility and endotoxin levels of freshly manufactured, individually vialled, and sealed CLP-PEG-MPC hydrogels were evaluated under Good Laboratory Practice (GLP) conditions by Eurofins Canada, a certified contract research laboratory.

2.2. Treatment with scCO₂-PAA, E-beam, and Chloroform

To test the bactericidal efficacy of E-beam (15 kGy)²² and scCO₂-PAA,²³ vials of individual corneal implants in 10 mL of 0.1 M PBS were inoculated with approximately 200 colony-forming units (CFU) of either Gram-positive *Staphylococcus aureus* (*S. aureus*) or Gram-negative *Pseudomonas aeruginosa* (*P. aeruginosa*) bacteria. For each bacterial strain, vials (*n* = 5) were either E-beam irradiated at a dose of 15 kGy (Nutek Bravo, Hayward, CA, USA) or treated with scCO₂-PAA (NovaSterilis, South Lansing, NY, USA) or with 1% chloroform in PBS (CF). Briefly, E-beam sterilization was performed on the optimized hydrogel formulation, both with and without bacterial inoculation per ISO13004:2022,²² under a quality management system compliant with ISO13485 and 21 CFR Part 820 GMP requirements.^{24,25} For scCO₂-PAA sterilization, the hydrogel samples were sterilized using the proprietary scCO₂-based NovaProcess developed by NovaSterilis Inc. For sterilization, the contents of each vial were transferred into a 30 mL amber glass bottle, and a PAA solution (NovaKill) was added to PBS to reach a final concentration of 30 ppm. The bottles were then capped with vented lids and individually sealed in 4.5" × 7" NovaPouches. Sterilization was performed using a 1.25 L NovaGenesis processing unit. Samples were then exposed to a scCO₂ atmosphere maintained at 10.34 MPa and 35 °C for a dwell time of 90 min. After processing, the samples were allowed to vent at room temperature inside a biosafety hood for 48 h. Residual PAA levels after 48 h as detected using the proprietary NovaPak method were below 0.4 ppm (*n* = 4). The vials containing the sterilized hydrogels were then aseptically recapped with nonvented lids, sealed with Parafilm, and placed in individual polymer pouches.

Two corresponding sterility tests were performed on the sterilized, bacteria spiked corneal hydrogel samples. In the first test, 100 μL of the PBS solution from each vial was streaked onto tryptic soy agar (TSA) plates, which were then incubated overnight at 37 °C to detect bacterial growth. In the second test, the implants were transferred into sterile containers containing 2 mL of tryptic soy broth (TSB) media (Teknova Inc., Hollister, CA, USA) and incubated overnight in a shaking incubator at 37 °C and 80 rpm. Samples from the TSB media were subsequently streaked onto TSA plates. After 24 h of incubation, the plates were examined, and CFU were counted to assess bacterial survival. The remaining hydrogels (no inoculation) were evaluated for their physicochemical and biological properties post-treatment.

2.3. Characterization of CLP-PEG-MPC Hydrogels after Sterilization

CLP-PEG-MPC hydrogels were characterized after fabrication and at 6 months post-fabrication after storage in a refrigerator at 4–8 °C, to obtain baseline values that would be attributable to storage, and to differentiate these from changes due to sterilization. Hydrogels were characterized again after sterilization to evaluate any changes that were due to E-beam or scCO₂-PAA treatment. Specific tests performed are discussed below.

2.3.1. Mechanical Properties. Rheological analysis was performed to measure the storage modulus (*G'*) of CLP-PEG-MPC hydrogels. A Discovery Hybrid-2 rheometer (DHR-2, TA Instruments, New Castle, DE, USA) fitted with a 20 mm circular geometry was used to characterize hydrogels that were 8 mm in diameter and 500 μm thick. A 0.1 N axial force was applied to each sample. An amplitude sweep was run from 0.1 to 150% using an angular frequency of 10 rad/s. Analysis was conducted using Trios v5.1.1.46572 (TA Instruments). Compression testing was also performed using a DHR-2 rheometer at 25 °C to evaluate hydrogel stability. Samples were compressed at a constant rate of 0.3 mm/min, and each group was tested in triplicate. The

compression modulus was calculated from the linear region of the resulting stress–strain curves.

2.3.2. Optical Properties. The transparency and diffuse reflectance of hydrogels (8 mm diameter, 0.5 mm thickness) post sterilization (without inoculation) with scCO_2 -PAA, E-beam (15 kGy), or chloroform were assessed using a custom-built setup. Spectrophotometric measurements were also used to record light transmission using a microplate reader (Tecan Spark, Switzerland) for stored (month 6) and baseline (day 0) samples. For each measurement, the hydrogels were placed in the wells of a transparent 96-well plate. The recorded optical density (OD) values were converted to optical transmission using the equation $T(\%) = 10^{-\text{OD}} \times 100$ where $\text{OD} = \text{OD}_{\text{hydrogel}} - \text{OD}_{\text{plate}}$. Finally, the average % light transmission (%) reported in Table 1 is calculated across the visible spectrum (380–780 nm). Five samples were measured.

2.3.3. Water Content. To determine the total equilibrated % water content (Wt), implants were immersed in $0.1\times$ PBS and weighed every 24 h until a constant weight was reached, indicating full hydration. The swollen weight (Ws) and dry weight (Wd) were recorded for each sample. The samples were then dried in an oven at 60°C until a stable dry weight (Wd) was obtained. The total equilibrated water content was calculated using the following formula:

$$\text{Wt}(\%) = \left[(\text{Ws} - \text{Wd}) / \text{Ws} \right] \times 100$$

2.3.4. Morphological Analysis. Morphological analysis of hydrogels poststerilization (E-beam and scCO_2 -PAA) was performed using field emission scanning electron microscopy (FE-SEM; JSM-7600F, JEOL Ltd., Tokyo, Japan) and compared with hydrogels stored in CF following lyophilization.

2.3.5. In Vitro Biocompatibility. Immortalized human corneal epithelial cells (HCEC) that retained characteristics of primary cells²⁶ were used to evaluate the *in vitro* biocompatibility of hydrogels after sterilization. Flat hydrogel sheets before and after sterilization were trephined into 6 mm diameter discs and placed in a 96-well tissue culture plate. Five thousand HCEC were seeded on top of each disc and supplemented with a serum-free medium (KSFM) containing 50 $\mu\text{g}/\text{mL}$ bovine pituitary extract, 5 ng/mL epidermal growth factor (Gibco-ThermoFisher, Grand Island, NY, USA), and 1% penicillin/streptomycin (Gibco) before placing in a 37°C incubator with 5% CO_2 . The cell culture medium was changed every other day. Alamar Blue (Sigma-Aldrich) staining was performed on day 1, day 3, and day 6 after cell seeding following the manufacturer's instructions. At each time point, 0.004% w/v resazurin sodium salt containing fresh media was added to the culture and incubated for 4 h. After the incubation, the cell-incubated resazurin sodium salt-containing medium was transferred to a new 96-well plate and read with a BioTek plate reader (Synergy 2, BioTek Instruments; Winooski, VT, USA) at 530/25 nm for excitation and 590/35 nm for emission. In addition, a live/dead cytotoxicity assay (Life Technologies Corporation, OR, USA) was performed on day 6, where cells were double-stained with calcein (Calcein AM) and ethidium homodimer-1 (EthD-1). Live cells and dead cells were stained as green and red, respectively. Images were captured by using a fluorescence microscope (EVOS M7000 Imaging System, Waltham, MA, USA). Cells grown on collagen hydrogels cross-linked with EDC/NHS and on tissue culture plastic (TCP) served as controls.²⁷

2.3.6. Assessment of Complement Activation via C3bc and sC5b-9. Isolation of whole blood from healthy human volunteers and incubations with serum were conducted in accordance with the guidelines of the Declaration of Helsinki and were approved by the Regional Ethical Review Board in Linköping, Sweden (Dnr 03-S20). Informed written consent was obtained from all human participants involved in the study. For complement activation study, 6 mm diameter pieces of non-bacteria spiked hydrogels were cut out with a trephine. Each sample was washed with 1 mL of sterile Dulbecco's Phosphate Buffered Saline with MgCl_2 and CaCl_2 (PBS; Sigma-Aldrich, Schnelldorf, Germany) in a 1.8 mL cryotube (Sarstedt, Nümbrecht, Germany). After removing the PBS, 400 μL of serum from a pooled sample of normal human serum (isolated from whole blood from five healthy donors) was added to each cornea.

In total, six sets of incubations were performed. The hydrogels were incubated at 37°C for 60 min in a water bath. After incubation, EDTA was added to a final concentration of 20 mM to prevent further activation. The serum was then transferred to new cryovials and stored at -80°C until immunoassays were performed. Three different types of controls were included for each set of hydrogels: (i) serum not incubated but immediately stopped with EDTA (T0), serving as a negative control, (ii) serum incubated in tubes without hydrogels (PBS) as a background control, and (iii) serum with zymosan A (Sigma-Aldrich, Schnelldorf, Germany) (200 $\mu\text{g}/\text{mL}$) as a positive control. Complement activation was assessed by quantifying C3 cleavage (i.e., generation of C3bc) and formation of the soluble terminal complement complex C5b-9 (sC5b-9) using ELISA.

2.4. Statistical Analyses

Data are reported as mean $\pm$ standard deviation. Graphs were prepared, and statistical analyses were performed using Prism 10 (GraphPad, USA). The normality of datasets was assessed using the Shapiro–Wilk test. For comparisons between two independent groups, an unpaired *t*-test was applied (Table 1). For multiple group comparisons, a one-way ANOVA was performed, followed by either Tukey's post hoc test or when normality or homogeneity of variance was not met, a nonparametric Kruskal–Wallis test with Dunn's multiple comparisons test. For two-way analyses, a two-way ANOVA with Tukey's post hoc test was conducted. The bars represent mean $\pm$ SD. Statistical significance was set at $p < 0.05$.

3. RESULTS

3.1. Assessment of CLP-PEG-MPC Hydrogels' Physical Properties

Interpenetrating networks of 25% CLP-PEG and 12% MPC were fabricated into hydrogels (Figure 1). Freshly prepared samples and those stored for 6 months at 4°C showed similar mechanical and physical properties (Table 1).

3.2. Sterilization Efficacy of E-beam vs scCO_2 -PAA

To establish a baseline before starting the sterilization experiments, the sterility and endotoxin levels of freshly manufactured, individually vialled, and sealed CLP-PEG-MPC hydrogels fabricated in an ISO7 cleanroom were evaluated under Good

Table 1. Stability of CLP-PEG-MPC Hydrogels over a 6-Month Storage in PBS^a

	Compressive strength (MPa)	Compressive modulus (kPa)	Fracture strain %	Storage modulus (kPa)	Water content %	Transmittance % (380–780 nm)
Day 0	1.76 ± 0.008	5.47 ± 1.34	78 ± 10.44	142.61 ± 14.75	78.79 ± 0.78	94.11 ± 5.51
Month 6	1.77 ± 0.008	4.76 ± 0.9	81.33 ± 8.14	156.8 ± 37.77	78.57 ± 0.12	84.74 ± 5.43

^aThe mechanical properties (compressive strength, compressive modulus, fracture strain, and storage modulus) and water content are presented as mean $\pm$ SD ($n = 3$). The average light transmittance (%) was measured across the visible spectrum (380–780 nm; $n = 5$). None of the comparisons were statistically significant.

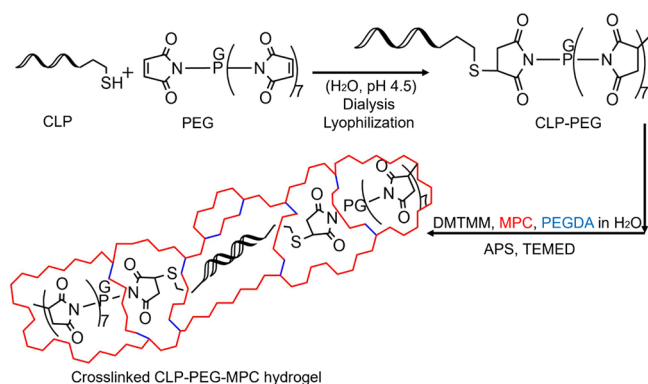


Figure 1. Chemical scheme showing the fabrication of CLP-PEG-MPC hydrogels.

Laboratory Practice (GLP) conditions by Eurofins Canada, a certified contract research laboratory. Endotoxin levels were 0.0012 endotoxin unit (EU)/mL ($n = 12$ in two separate batches). This level was well below the accepted level of <0.5 EU/mL limit established for implantable medical devices.²⁸ The implants were deemed sterile as no microbial growth was detected ($n = 16$ in two separate batches).

As the implants were sterile, in order to determine sterilization efficacy, individually vialled CLP-PEG-MPC samples were spiked with a known bacterial load of 200 CFU of either *P. aeruginosa* or *S. aureus* and sealed prior to sterilization.

After sterilization, no detectable CFU for either bacteria species was observed in either the storage media or implants treated with scCO_2 -PAA (Figure 2). However, complete

eradication of bacteria was not observed in E-beam or chloroform (CF)-treated samples. For *P. aeruginosa* inoculated groups, while 80% of the implants were free of bacteria for both E-beam and CF treatments, live bacteria were found in the storage media of all samples in both these groups. The CFU of bacteria found in these implant samples are shown in Figure 2A,B.

For *S. aureus* inoculated groups, 80% of the E-beamed implants were bacteria free. However, only 40% of CF-treated implants were bacteria free. Analysis of the PBS storage media showed that 60% of E-beamed vials and 40% of the CF vials were *S. aureus* free. The CFU of bacteria found in these samples are shown in Figure 2C,D.

Implant and storage media bioburden from treated samples were compared to the untreated PBS samples with a spiked initial concentration (IC) of 200 CFU of each bacterium. For the implants, there was significantly lower bioburden in scCO_2 -PAA for *P. aeruginosa* ($p = 0.0008$) and *S. aureus* ($p = 0.0006$) when compared to IC samples (Figure 2A,B). For the storage media, the scCO_2 -PAA sterilized samples showed complete eradication for both bacteria (Figure 2C,D).

3.3. Sterilization Effects on CLP-PEG-MPC Hydrogel Properties

Flat hydrogels, without bacterial inoculation, were evaluated for the effects of sterilization on their physical, optical, and mechanical properties. The storage moduli for scCO_2 -PAA, E-beam, CF, and non-sterilized PBS treatments were 243.27 ± 23.95 , 185.99 ± 22.90 , 235.83 ± 21.46 , and 252.23 ± 12.78 kPa, respectively. No significant differences were observed among scCO_2 -PAA, CF, and PBS treatments. However, the storage moduli of E-beamed samples were significantly decreased compared to those treated with scCO_2 -PAA ($p = 0.02$) or untreated in PBS ($p = 0.004$) (Figure 3A). White light transmissions (over the visible spectrum) through samples treated by scCO_2 -PAA, E-beam, CF, or left untreated in PBS were $88.74 \pm 3.04\%$, $87.48 \pm 3.18\%$, $91.07 \pm 2.02\%$, and $90.47 \pm 2.11\%$, respectively. Additionally, the average diffuse reflectance for scCO_2 -PAA, E-beam, CF, and PBS was $0.71 \pm 0.21\%$, $0.80 \pm 0.09\%$, $0.75 \pm 0.11\%$, and $0.73 \pm 0.09\%$, respectively. No significant difference was observed in the optical properties between scCO_2 -PAA and E-beam-treated samples (Figure 3B,C). FE-SEM imaging of lyophilized CLP-PEG-MPC hydrogels showed that E-beam irradiation resulted in surface alterations characterized by large, irregular pores and a textured surface (Figure 3D). scCO_2 -PAA or CF-treated hydrogels, however, retained their smooth, minimally textured surfaces that were similar to the PBS control.

The effect of different sterilization methods on CLP-PEG-MPC biocompatibility was examined by the capacity of the differentially treated hydrogels to support proliferation of immortalized human cornea epithelial cells (HCEC). Fluorescence imaging of live cells (green) revealed that all the treated hydrogels (scCO_2 -PAA, E-beam, and CF) supported HCEC growth over 6 days of culture (Figure 4A,B). HCEC seeded on collagen hydrogels and tissue culture plastic (TCP) served as controls of cell health and viability. Quantitative analysis of the relative fluorescence units (RFU) from cells of different substrata compared to those grown on TCP was measured (Figure 4B). On day 1, while there were no significant differences in RFU among all the sterilized groups, a significant difference ($p < 0.0001$) was detected between cells growing on scCO_2 -treated CLP-PEG-MPC hydrogels and those grown on collagen.

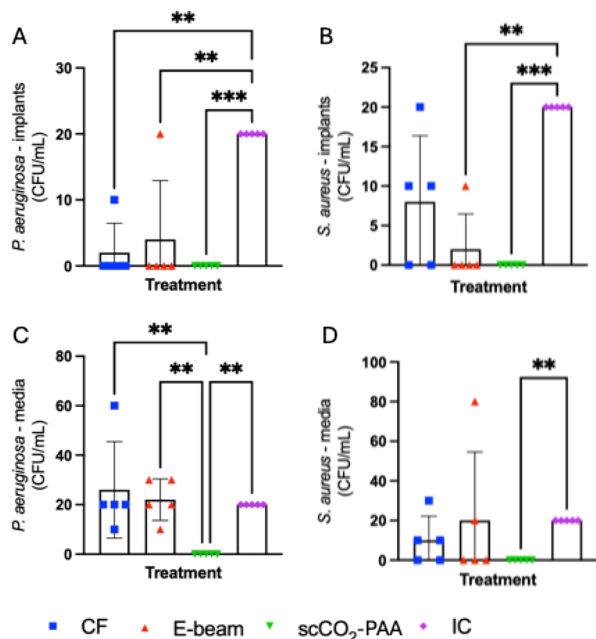


Figure 2. Quantitative analysis of bacterial growth from the remaining bioburden in CLP-PEG-MPC implants treated with scCO_2 -PAA, E-beam, and chloroform (CF) after spiking *P. aeruginosa* and *S. aureus*. The bacterial growth was compared to that of samples stored in untreated PBS with an initial spiking concentration (IC) of 200 CFU bacteria. Bacterial growth from implant samples (A, B) and PBS storage media (C, D) was compared. $N = 5$ for all groups.

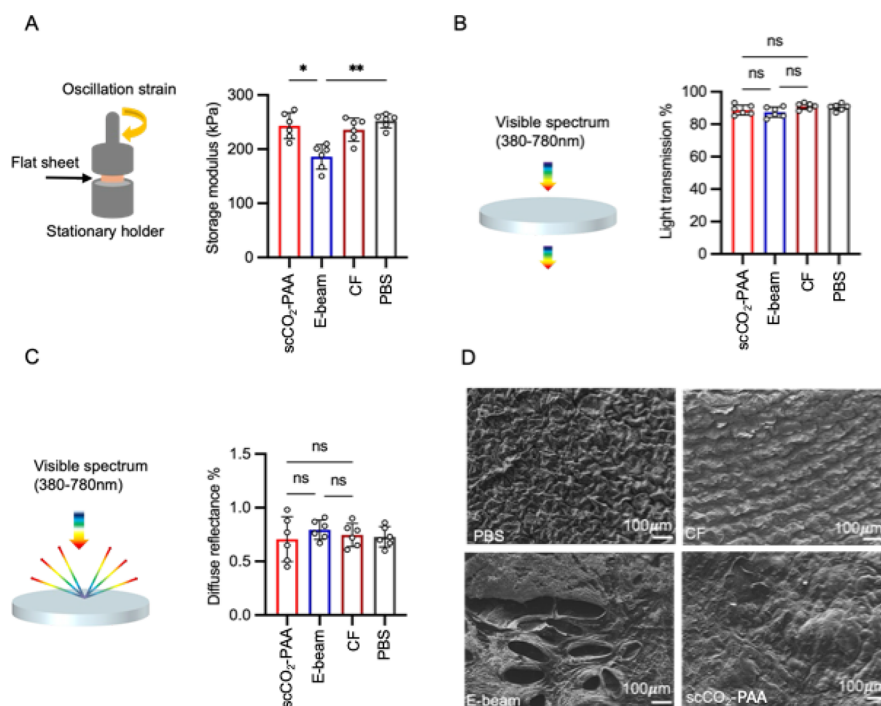


Figure 3. Effect of scCO₂-PAA, E-beam, and chloroform (CF) sterilization on the mechanical, optical, and morphological properties of CLP-PEG-MPC hydrogels stored in PBS. Control samples remain unsterilized in PBS. (A) Mechanical properties of hydrogels showing the differences in storage modulus (kPa) in the linear viscoelastic region as measured by rheometry. Controls were unsterilized ($n = 6$). (B) Percentage transmission of white light (visible spectrum) for the different groups ($n = 6$). (C) Diffuse reflectance (%) of white light (visible spectrum) for the different groups ($n = 6$). (D) SEM analysis of corneal implants after the different sterilization techniques compared to the unsterilized PBS control; scale bars, 100 μm . Asterisks indicated statistical significance; ns, nonsignificant.

By 6 days, small but significant reductions were observed between cultures on scCO₂-PAA and CF or E-beam-treated hydrogels in addition to those on collagen ($p < 0.0001$). Together, these results demonstrate that while all sterilization methods preserved hydrogel biocompatibility, scCO₂-PAA treatment resulted in comparatively lower HCEC proliferation over time compared to E-beam and CF sterilization.

3.4. Complement Activation in Contact with Human Serum

The complement system has an important role in the destruction and removal of pathogens and cell debris.²⁹ The C3 complement proteins, when activated, bind to pathogens and tag them for destruction by immune cells through a process known as opsonization. The T0 control condition (with non-incubated serum) showed minimal C3bc formation as expected (Figure 5A). Serum incubated with or without corneal implants showed elevated C3bc concentrations, indicating complement activation. Importantly, the C3bc levels in all groups containing corneal implants were not significantly higher than the background control (PBS). This indicated that the incubation itself induced C3 cleavage, but the contribution from the cornea implants was only minor or absent and did not differ among sterilization methods. Zymosan, a yeast cell wall derivative often used to stimulate immune responses, served as a positive control. Zymosan showed the highest level of C3bc (340.68 ± 70.62 complement activating units (AU) per mL), confirming the assay's responsiveness and robust complement activation with significant difference compared with E-beam and scCO₂-PAA ($p < 0.0001$). Zymosan triggered strong formation

(>250 AU/mL) of sC5b-9, a protein complex formed by the activation of the complement system, validating the responsiveness of the assay. The T0 negative control showed negligible sC5b-9 formation, while the PBS-only group showed minimal activation, indicating a low background (Figure 5B). Implants that were non-sterilized (stored in PBS only), E-beamed, and scCO₂-PAA treated had elevated sC5b-9 levels, suggesting activation of the terminal complement cascade. However, the non-sterilized PBS control implants triggered the highest activation (32.02 ± 3.36 AU/mL), while sterilized implants (E-beam: 15.07 ± 1.59 ; scCO₂-PAA: 13.59 ± 1.47) induced lower activation levels, indicating a potential benefit of sterilization in reducing complement activation. Statistical analysis of sC5b-9 across groups showed significant differences ($p = 0.03$) between the PBS only control and unsterilized implants in PBS. Most notably, however, are the differences between non-sterilized implants in PBS and the E-beam and scCO₂-PAA sterilized samples.

4. DISCUSSION

Our fully synthetic CLP-PEG-MPC implants had promoted rapid and stable regeneration of corneal tissues and nerves in pre-clinical animal models^{10,11} meriting further evaluation in clinical trials. However, ensuring sterility of these corneal implants is crucial to meet the hygienic standards required for critical medical devices, as outlined in the Medical Device Regulation (EU 2017/745, MDR).³⁰ For hydrogels to be suitable for clinical use and obtain regulatory approval, they should be sterile or aseptic to ensure patient safety without compromising their functional characteristics.³¹ To ensure the feasibility of preparing the

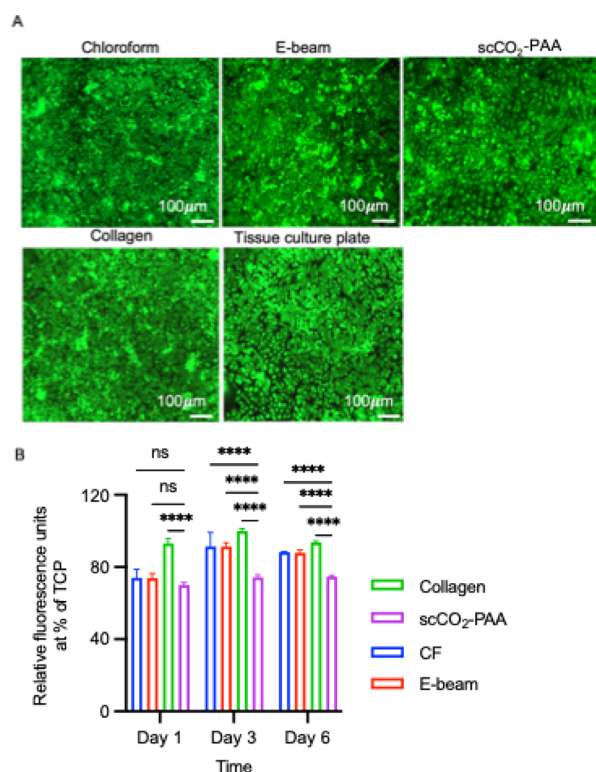


Figure 4. Effects of scCO₂-PAA, E-beam, and chloroform (CF) sterilization on the biological properties of CLP-PEG-MPC hydrogels. (A) Viability (green fluorescence) of HCEC cells cultured on CLP-PEG-MPC hydrogels that were sterilized by scCO₂-PAA, E-beam, and CF, compared to those grown on control collagen and tissue culture plastic (TCP) substrates. (B) Quantitative analysis of the relative fluorescence units over 6 days of HCEC grown on the different substrates as a % of cells grown on TCP ($n = 3$). Asterisks indicated statistical significance ($p \leq 0.05$).

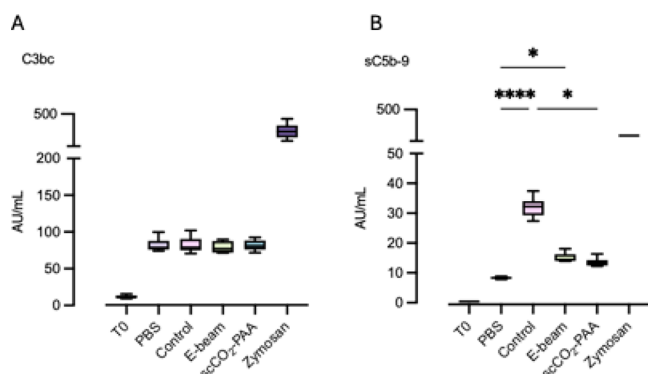


Figure 5. (A) Assessment of complement activation by C3bc and (B) sC5b-9 quantification in serum incubated with corneal implants at 37 °C for 60 min, $n = 6$. Controls included serum immediately stopped with EDTA (T0, negative control), serum incubated without corneas (PBS, background control), and serum incubated with zymosan (positive control). Data represent the degree of complement activation, measured as complement activating units (AU), triggered by the implants relative to controls.

hydrogels in our laboratory (Montreal, Canada), transporting them to a collaborating lab for spiking in the United States, and subsequently sending them to external facilities for scCO₂-PAA or E-beam sterilization, it was crucial that the samples maintained their structural integrity throughout these steps. Although the entire process was expected to take less than 6 months, a 6-month stability assessment was performed to confirm that the hydrogels retained their structural properties during extended storage. The stability results showed no significant changes in the physical or mechanical properties of CLP-PEG-MPC hydrogels over 6 months of storage and closely resembled the native human cornea in both water content and optical transmittance.^{32,33}

In the present study, none of the sterilization techniques altered the optical properties of the hydrogels, which remained optically clear compared to native cornea.³⁴

While transparency was unchanged, E-beam irradiation at 15 kGy altered the hydrogel's mechanical properties (storage modulus), in keeping with reports of E-beam altering the structure and properties of biomaterials by inducing cross-linking and/or degradation of the collagen matrix.³⁵ In our previous study, E-beam irradiation of RHCI-III-MPC cross-linked with EDC did not significantly alter the optical or mechanical properties when compared to unirradiated controls.⁸ It has been reported that EDC provides radioprotective effects during E-beam irradiation,³⁶ or the full-length RHCI-III may have been more resistant to the irradiation. In the present study, where DMTMM was used as the primary cross-linking agent, the rheological properties of E-beamed hydrogels decreased significantly compared to hydrogels stored at 1% CF and 0.1 M PBS. However, it should be noted that the storage modulus of CLP-PEG-MPC hydrogels in Table 1 is not the same as that in Figure 3, although within the general range of a 100 kPa. The differences are most likely due to batch and lot differences of the raw materials used in implant fabrication.

E-beam irradiation is a form of ionizing radiation that can induce cross-linking between polymer chains, but it may also degrade the polymer structure by cleaving bonds within the polymer backbone.^{37–39} DMTMM cross-links by forming stable ester bonds with hydroxyl and amine groups in the polymer. However, ionizing radiation can break these ester bonds between DMTMM and the polymer chains, which could explain the observed changes in mechanical properties. E-beam exposure appeared to result in the formation of smaller pores in the hydrogel as a result of extra cross-links induced by the irradiation.⁴⁰ Here, cross-linking that shrinks pore size could have resulted in a change of the surface texture of CLP-PEG-MPC hydrogels noted here.

In contrast, scCO₂ sterilization with PAA preserved the original morphology of the hydrogel due to the mild operational conditions. scCO₂-PAA is a chemically based sterility technique operating under mild conditions, with low temperature and pressure, acting as a solvent to sterilize materials without inducing high-energy changes.¹⁸ A previous study also showed that mechanical properties of polysaccharide- and collagen-based biomaterials were less affected by scCO₂ sterilization compared to traditional methods.¹⁵ Russell et al. found no significant differences in the mechanical properties of bone tissues after scCO₂⁴¹ treatment, while Bednarski et al. showed that scCO₂ treatment with NovaKill preserved tendon collagen morphology and ECM structure, unlike gamma irradiation, which caused collagen separation and ECM degradation.⁴² Similarly, we found no significant changes in the measured storage modulus, indicating that scCO₂-PAA most likely did not cause

alterations to the bonds in the cross-linked structure of the hydrogel. Nevertheless, we noted in Figure 4 that despite the overall advantages noted over E-beam and CF, scCO₂-PAA-treated hydrogels showed a small but statistically significant decrease in the rate of HCEC proliferation compared to the other methods.

As our manufacturing process in an ISO7 cleanroom was sterile with no microbial contamination, it was not possible to determine a sterilization dose in accordance with ISO11737-1:2018,⁴³ which requires a measurable bioburden for dose establishment. We therefore intentionally inoculated the implants with a known bacterial load. The process of scCO₂-PAA requires PAA to meet sterilization standards and to enhance sterilization effectiveness.^{44,45} It has been suggested that the low levels of PAA used in scCO₂-PAA sterilization do not induce degradation.⁴² scCO₂-PAA's ability to penetrate even the smallest pores is advantageous when combined with PAA for the effective inactivation of contaminating microorganisms⁴⁴ as seen in our results where all bacteria were inactivated in both the storage media and the implants.

On the other hand, E-beam irradiation at 15 kGy did not completely eradicate the spiked bacteria from CLP-PEG-MPC implants and their storage media. While this observation differs from our previously published report for RHCIII-MPC implants, we note that the previous study used a higher dose of E-beam irradiation at 17 kGy.⁸ Here, we selected a lower dose of 15 kGy of irradiation as the 17 kGy irradiated implants had shown sensitivity to high levels of collagenase compared to unirradiated controls and also showed slower cell growth.⁸ E-beam radiation works by breaking molecular bonds within the target material and microbial cells. It does this by transferring energy to the atoms that can lead to disruption of DNA and cellular metabolism, effectively killing microorganisms.⁴⁶ However, at lower doses like 15 kGy tested in this study, the radiation may not deliver enough energy to disrupt all microorganisms thoroughly. This could explain why some bacteria remained active after E-beam sterilization. To conclusively determine the efficacy of E-beam to sterilize CLP-PEG-MPC implants beyond this initial comparison, however, a dose response study will be needed.

One advantage of E-beam over scCO₂-PAA is that capped vials are sterilized without further manipulation. The current limitation of the scCO₂-PAA sterilization process is the need for the change of the lids of each sample-containing vial. The vented lids used to cap vials containing the hydrogels for the sterilization process had to be aseptically replaced with non-vented lids to seal each vial for shipping or storage, preventing this technique from being equivalent to sterilization in a sealed final-use package.

Sterilization using CF closely matched the E-beam sterilized samples. CF has been reported to have antibacterial activity.⁴⁷ However, it is toxic and hence, its use entails the need for thorough washing to remove traces prior to the use of implants for surgery. As such, CF is not a convenient means of terminal sterilization.

The complement system, a central component of innate immunity, can mediate corneal graft rejection through the generation of inflammatory effector molecules.^{48,49} Complement activation induced by the tested corneal implants was generally low. C3 cleavage did not exceed background control levels. However, terminal pathway activation, as indicated by sC5b-9 formation, was elevated 4-fold in the untreated control corneal implants but was reduced by more than 50% following sterilization. The underlying mechanism cannot be resolved within the

current experimental design because only soluble C3bc and sC5b-9 were measured. However, future studies should assess surface-bound complement components, surface-associated complement convertase formation, and the binding of endogenous complement-regulatory proteins. Additional preclinical evaluations in rabbit or pig models will be necessary to investigate the impact of both sterilization and long-term storage on corneal regeneration outcomes to further validate the clinical potential of these implants.

5. CONCLUSIONS

Sterilization with scCO₂-PAA was able to inactivate the low-level inoculated bioburden without compromising structural, optical, or mechanical properties. E-beam sterilization at 15 kGy, although partially effective, induced structural changes and altered the mechanical properties. These findings suggest that for high-water-content hydrogels such as CLP-PEG-MPC developed as implants, scCO₂-PAA is a promising sterilization option and further research is merited. Future work should focus on validating the sterility and regenerative capacity of these implants after long-term storage in preclinical rabbit or pig models, ultimately supporting their translation to human clinical trials.

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Notes

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ABBREVIATIONS

CF	chloroform
CFU	colony-forming units
CLP	collagen-like peptide
PEG	polyethylene glycol
MPC	poly(2-methacryloyloxyethyl phosphorylcholine)
scCO ₂	supercritical carbon dioxide
DMTMM	4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride
e-beam	electron beam
HCEC	human corneal epithelial cells
PAA	peracetic acid
RHCIII	recombinant human collagen type III

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