Published in final edited form as:

Macromol Biosci. 2016 June ; 16(6): 836–846. doi:10.1002/mabi.201500410.

The effect of sterilization methods on the structural and chemical properties of fibrin microthread scaffolds

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Abstract

A challenge for the design of scaffolds in tissue engineering is to determine a terminal sterilization method that will retain the structural and biochemical properties of the materials. Since commonly used heat and ionizing energy-based sterilization methods have been shown to alter the material properties of protein-based scaffolds, we evaluate the effects of ethanol and ethylene oxide (EtO) sterilization on the cellular compatibility and the structural, chemical, and mechanical properties of uncrosslinked, UV crosslinked, or carbodiimide crosslinked fibrin microthreads in neutral (EDCn) or acidic (EDCa) buffers. EtO sterilization significantly reduces the tensile strength of

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Disclosures: G.D.P. discloses that he is a co-founder and has an equity interest in Vitathreads L.L.C., a company that has licensed intellectual property associated with fibrin microthreads.

uncrosslinked microthreads. Surface chemistry analyses show that EtO sterilization induces alkylation of EDCa microthreads leading to a significant reduction in myoblast attachment. The material properties of EDCn microthreads do not appear to be affected by sterilization method. These results significantly enhance the understanding of how sterilization or crosslinking techniques affect the material properties of protein scaffolds.

Graphical abstract

The structural and chemical properties of fibrin based scaffolds are affected by crosslinking and sterilization protocols. Ethanol and ethylene oxide gas sterilization procedures were performed on uncrosslinked, carbodiimide or ultraviolet light crosslinked fibrin microthreads to evaluate the mechanical, structural, and chemical properties of these materials. The data presented here inform the strategic design of fibrin based scaffolds.

Keywords

sterilization; microthreads; scaffolds; biomaterials; fibrin

1. INTRODUCTION

A common challenge for all biopolymer-based scaffolds in the field of tissue engineering is to identify a method of terminal sterilization that will not negatively affect the material's structural or biochemical properties. Heat-based sterilization methods, such as autoclaving, often denature protein-based materials, compromising the mechanical function of these materials.^[1] Other sterilization methods, such as electron beam irradiation and gamma irradiation, expose materials to ionizing energy to destroy genetic material, but the high energies delivered in these methods have been found to degrade protein-based scaffolds as well.^[2–5] Chemical based sterilization methods, such as ethylene oxide (EtO), are becoming increasingly common in the medical device industry because their low processing temperatures and non-ionizing methods of sterilization minimize the degradation of biopolymer scaffolds. The toxicity of residual EtO vapors remains a concern for its

widespread usage,^[6] and any EtO sterilized scaffold is typically aerated for at least one day to remove any residual EtO molecules prior to implantation. Ethanol (EtOH) is another common chemical based sterilization method used in the laboratory. Because EtOH may not destroy bacterial endospores (spores), it is considered a germicidal or disinfection technique rather than a sterilization method. However, EtOH disinfection has been repeatedly shown to be effective, despite this limitation.^[7, 8] While EtOH disinfection is commonly used in benchtop experiments for the characterization of cell-material interactions, EtO is routinely used for production-scale sterilization of commercially manufactured biomaterials and medical devices.^[9] As such, it is imperative to understand how different sterilization techniques affect the structural and chemical properties of these materials to enable the strategic design of a scaffolding material for specific applications.

Fibrin is a branched, microfibrillar polymer that is deposited as a provisional matrix during the initial stage of wound healing, which functions to stabilize the injury site and to guide endogenous tissue regeneration. Because of its ability to direct wound healing, fibrin has been extensively used as a scaffold material for tissue engineering of a variety of tissue systems such as skeletal muscle,^[10–13] blood vessels,^[14–17] cardiac tissue,^[17–20] and nervous tissue.^[21, 22] Fibrin is formed when activated thrombin cleaves fibrinopeptide A and B from fibrinogen monomers, allowing fibrinogen to self-assemble into a complex fibrillar network.^[23] This self-directed polymerization allows fibrin to be cast into hydrogels with a variety of forms, as well as into more discrete structures such as microthreads.

Fibrin microthreads combine the advantageous biochemical properties of fibrin-based hydrogels with an aligned fibrous architecture and are used to direct cell-mediated tissue regeneration, target localized delivery of growth factors or cells, or suture tissues that require sustained uniaxial loading. Fibrin microthreads have discrete, tunable mechanical and structural properties that facilitate cell alignment along the longitudinal axis of the scaffolds.^[24] We investigated the use of ultraviolet (UV) light and carbodiimide crosslinking, to both enhance the mechanical properties of fibrin microthreads and to slow the rate of proteolytic degradation.^[10, 25] Increases in the time of UV exposure strengthened the tensile properties of fibrin microthreads until 1 hour of exposure, at which point there was a qualitative decrease in the mechanical properties.^[25] Additionally, by controlling the buffer environment of the carbodiimide reaction, we developed materials with varied mechanical properties and degradation profiles.^[10] These crosslinking methods enable the design of microthread-based scaffolds for tissue engineering applications with varied structural and mechanical requirements.

To investigate the effects of terminal sterilization processes on fibrin-based scaffolds, we systematically analyzed the mechanical and biochemical properties of crosslinked fibrin microthreads. Uncrosslinked (UNX) microthreads and microthreads that were UV crosslinked for 40 minutes (UV40) or carbodiimide crosslinked in either acidic (EDCa) or neutral (EDCn) buffers were treated with either EtO or EtOH. We compared the mechanical properties of these materials, evaluated the surface chemistry of the scaffolds and assessed the attachment and proliferation of myoblasts on each of the scaffolds. The results show that while EtOH disinfection does not significantly affect the mechanical or chemical properties of UNX microthreads, EtO sterilization decreases the mechanical properties of UNX

microthreads as well as cell attachment to EDCa microthreads. In contrast, the mechanical strength and biochemical properties of EDCn microthreads do not appear to be affected by either EtO or EtOH treatments. When evaluating the surface chemistry, the EDCa microthreads that were EtO sterilized showed an increase in bands associated with C-H stretching, suggestive of an increase in alkylation. This increased alkylation may offer an explanation as to why cell attachment was decreased for this group. Understanding the impact of crosslinking and sterilization procedures on fibrin-based scaffolds will inform the design of biopolymer scaffolds that will be used as implantable medical devices.

2. MATERIALS and METHODS

2.1. Fibrin Microthread Preparation

2.1.1. Microthread Extrusion—Fibrin microthreads were co-extruded from solutions of fibrinogen and thrombin using extrusion techniques described previously.^[10] Briefly, fibrinogen from bovine plasma (MP Biomedicals, Santa Ana, CA; Cat. No. 820224) was dissolved in HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) buffered saline (HBS, 20 mM HEPES, 0.9% NaCl; pH 7.4) at 70 mg mL⁻¹ and stored at -20 °C until use. Thrombin from bovine plasma (Sigma; T4648) was dissolved in HBS at 40 U mL⁻¹ and stored at -20 °C until use.

To fabricate microthreads, fibrinogen and thrombin solutions were thawed and warmed to room temperature. Thrombin was mixed with a 40 mM CaCl₂ (Sigma) solution to form a 6 U mL⁻¹ working solution and equal volumes of fibrinogen and thrombin/CaCl₂ solutions were loaded into separate 1 mL syringes, which were inserted into a blending applicator tip (Micromedics Inc., St. Paul, MN; SA-3670). The solutions were combined in the blending applicator and extruded through polyethylene tubing (BD, Sparks, MD) with an inner diameter of 0.86 mm into a bath of 10 mM HEPES (pH 7.4) in a Teflon coated pan at a rate of 0.225 mL min⁻¹ using a dual syringe pump. Microthreads were incubated in 10 mM HEPES buffer for 10 minutes to facilitate fibrin polymerization, at which point the microthreads were removed from the buffer solution and stretched to approximately 150% their original lengths to form three 19 cm microthreads.

2.1.2. EDC Crosslinking Procedure—Fibrin microthreads were crosslinked with carbodiimide chemistry using techniques described previously.^[10] Briefly, microthreads were hydrated in acidic buffer (50 mM MES, Sigma; pH 5.2) or neutral buffer (100 mM NaH₂PO₄, Sigma; pH 7.4) for 30 minutes and then crosslinked with either acidic (EDCa) or neutral (EDCn) buffer containing 16 mM N-hydroxysuccinimide (NHS, Sigma) and 28 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, Sigma) for 2 hours at room temperature. After crosslinking, the buffered EDC/NHS solution was aspirated and the microthreads were rinsed three times in (de-ionized) DI water, air dried, and stored in a desiccator until use.

2.1.3. UV Crosslinking—Fibrin microthreads were crosslinked with ultraviolet (UV) light using techniques described previously.^[25] Briefly, microthreads were secured on a reflective aluminum foil surface that was centered 11 cm from a bank of 5–8 W UV tubes emitting at a primary wavelength of 254 nm in a model CL-1000 ultraviolet crosslinker

(UVP, Upland, CA) for 40 minutes at an exposure setting of $120,000 \mu\text{J cm}^{-2}$ and stored in a desiccator until use.

2.2. Sterilization Methods of Crosslinked Fibrin Microthreads

2.2.1. Ethanol Disinfection—Microthreads were rehydrated in DI water for 30 minutes and then incubated in 70% ethanol (EtOH) in a biosafety cabinet for 90 minutes. Samples were rinsed three times in sterile DI water, air dried, and stored in a desiccator until use.

2.1.2. Ethylene Oxide Sterilization—Dry scaffolds were sterilized by exposure to ethylene oxide gas (EtO) for 12 hours using an Anprolene AN74i gas sterilizer (Anderson Products, Inc., Haw River, NC), aerated for at least 24 hr to remove residual EtO molecules, and stored in a desiccator until use.

2.3. Mechanical Characterization of Sterilized Fibrin Microthreads

Mechanical characterization of sterilized fibrin microthreads was performed using techniques described previously (Figure 1A).^[10] Briefly, individual microthreads were affixed with medical grade silicone adhesive to vellum paper frames with precut windows that defined the region of loading. An initial gauge length of 2.0 cm was defined as the distance between adhesive spots at the edges of the precut window in the vellum frame (Figure 1B). The microthreads on the vellum frames were hydrated in phosphate buffered saline (PBS) for at least 60 minutes prior to testing. Hydrated microthread diameters were measured using a calibrated reticule with a 10X objective, coupled to a Nikon Eclipse E600 upright microscope (Melville, NY). Microthreads were assumed to be cylindrical, and the diameter was determined by averaging 3 measurements taken along the length of each microthread to define the cross-sectional area. After hydration, microthreads were securely mounted in the grips of a uniaxial testing machine (ElectroPuls E1000; Instron, Norwood, MA) and a 1 N load cell (Figure 1A), the edges of each vellum frame were cut (Figure 1C), and the microthreads were uniaxially loaded until failure at a 50% strain rate (10 mm min^{-1}) (Figure 1D). Force and displacement were recorded continuously throughout each test at a frequency of 10 Hz. The mechanical failure load of each microthread was recorded as the point where a rapid (80%) drop from the maximum load occurred. The grip-vellum paper interface was visually inspected during each test to ensure that no slippage occurred. Microthreads that slipped in the grips during testing were excluded from further analysis. Engineering stress was calculated as the amount of force detected by the load cell divided by the initial cross-sectional area. Strain was calculated as the increased extension from the gauge-length.

A MATLAB (MathWorks, Natick, MA) script was written to analyze the ultimate tensile strength (UTS), maximum tangent modulus (MTM), strain at failure (SAF), and load at failure for each sample. The MTM was defined as the highest linear region in the stress-strain curve for each sample over a moving window length (corresponding to 20% of the total length of each test) and fitted to a linear region of the stress-strain curve. Statistical analysis was used to identify and remove outliers as defined by microthreads whose wet diameters were 1.5 times greater than the inner quartile range (IQR) for each sample set (average wet diameter $\pm 1.5 \cdot \text{IQR}$). To ensure this method was robust, we determined that

the distribution of wet diameter measurements was normal using SigmaPlot 11.0 software (Systat Software, Inc., San Jose, CA) to validate our definition, and exclusion, of outliers. Data points falling outside of this range were excluded from later statistical analyses.

2.4. Structural Characterization of Stretched Fibrin Microthreads

Prior to mechanical testing, the diameters of dry crosslinked microthreads were recorded using a calibrated reticule with a 10X objective. In each case, the diameter for each microthread was averaged from three measurements taken along the length. The wet diameter measurements taken during the mechanical testing procedure were also used to calculate the swelling ratio which was defined as the ratio of the wet diameter of a microthread to its dry diameter (wet/dry diameter).

2.5. FT-IR Characterization

Eight crosslinked, EtO or EtOH treated microthreads were cut into 1–2 mm long fragments and were positioned onto the attenuated total reflectance (ATR) crystal of a Bruker Vertex 70 instrument (Bruker, Billerica, MA) with a Golden Gate ATR accessory (Specac, Swedesboro, NJ). FT-IR measurements of the absorbance spectra were collected in the mid-IR range (4000–700 cm^{-1}). Backgrounds were subtracted from each spectrum. For each condition, 3 separate batches of sterilized microthreads were averaged together to obtain a representative spectrum for comparison to other conditions. For quantitative comparison of amides, ratios of amide I ($\sim 1630 \text{ cm}^{-1}$) to C-H stretch ($\sim 2955 \text{ cm}^{-1}$), and amide II ($\sim 1520 \text{ cm}^{-1}$) to C-H stretch ($\sim 2955 \text{ cm}^{-1}$) for each sample were compared and statistically evaluated.

2.6. Cell Culture

Immortalized mouse myoblasts (CRL-1772, ATCC, Manassas, VA; C2C12) were cultured in a 1:1 (v/v) ratio of high glucose Dulbecco's modified Eagle Medium (DMEM, Gibco BRL, Gaithersburg, MD) and Ham's F12 (Gibco), supplemented with 4 mM L-glutamine and 10% fetal bovine serum (FBS, HyClone, Logan, UT). Cells were incubated at 37 °C with 5% CO_2 and maintained at a density below 70% confluence using standard cell culture techniques. Routine cell passage was conducted using 0.25% trypsin-EDTA (CellGro, Manassas, VA).

2.7. Cell Attachment Assay

2.7.1. Microthread Bundling and Seeding—To facilitate cell seeding for cell attachment assays, microthreads were bundled together in groups of 10 and adhered to either stainless steel rings (Seastrom Manufacturing, Twin Falls, ID; inner diameter, 0.750 inches; outer diameter, 1.188 inches; thickness, 0.005 inches) or custom made polydimethylsiloxane rings (PDMS, Ellsworth Adhesives, Germantown, WI; inner diameter, 0.75 inches; thickness, 0.15 inches) with medical grade silicone adhesive. Microthread bundles secured to stainless steel rings were disinfected in 70% ethanol for 90 minutes, rinsed 3 times in DI water, air dried in a biosafety hood, and stored in a desiccator until use. Microthread bundles secured to PDMS rings were sterilized with EtO, aerated for at least 24 hrs to remove residual EtO, and stored in a desiccator until use. Individual sterilized rings were placed in

wells of a standard six-well plate over an elevated 13 mm diameter circular Thermanox™ coverslip (Nalge Nunc International, Rochester, NY), as described previously.^[26]

Immediately prior to seeding, 150 μ L of sterile PBS was added to the coverslip to hydrate the microthreads for at least 1 hour. To attach C2C12 cells to the microthread bundles, the PBS was aspirated and replaced with 100 μ L of cell suspension (100,000 cells mL^{-1}) in complete culture medium. After a four-hour incubation, cell-seeded microthread bundles were transferred into a new six-well plate with 2 mL of fresh medium and returned to the incubator. Each experimental treatment was run in duplicate and medium was changed every 2 days.

2.7.2. Analysis of Cell Number—Quantification of the cell density of C2C12 cells on microthread bundles was determined after 1 or 3 days of culture. At each time point, cell-seeded microthread bundles were fixed in 4% paraformaldehyde for 15 minutes and rinsed 3 times in DI water. Constructs were stained with Hoechst 33342 (1:6000, Molecular Probes) for 6 minutes and rinsed 3 times with DI water. Three regions of the cell-seeding area were visualized on a Leica inverted microscope using a 10X objective and photomicrographs taken using Leica imaging software and images of nuclei were counted using ImageJ. To calculate cell density on the surface of the microthread bundles, it was assumed that images corresponded to one half of the surface area of the microthread bundle. Approximating a cylindrical geometry for each bundle, length (l) and diameter (d) measurements enabled calculation of the total surface area (SA) as: $SA = 0.5 * \pi * l * d$. Total cell number was divided by the SA of each image to calculate the cell surface density.

2.8. Statistical Analyses

Statistical analyses were performed using a one-way analysis of variance (ANOVA) with $p < 0.05$ indicating significant differences between groups, using SigmaPlot 11.0 software. For post-hoc analyses, Holm-Sidak pairwise multiple comparison tests were performed to determine significant differences between experimental groups using an overall significance level of $p < 0.05$. Where indicated, a Student's t-test was performed with $p < 0.05$ indicating significant differences between groups. The data are reported as means $\pm$ standard deviation for the mechanical and structural characterization and as means $\pm$ standard error for the cell attachment studies.

3. RESULTS and DISCUSSION

3.1. Sterilization Methods Affect the Mechanical Properties of Fibrin Microthreads

The mechanical properties of crosslinked fibrin microthreads treated with either ethanol (EtOH) or ethylene oxide (EtO) were investigated to evaluate the effects of these sterilization methods on fibrin microthread scaffolds. Characteristic stress-strain curves for uncrosslinked (UNX, Figure 2A), UV crosslinked (UV40, Figure 2B), carbodiimide crosslinked in neutral buffer (EDCn, Figure 2C), or carbodiimide crosslinked in acidic buffer (EDCa, Figure 2D) fibrin microthreads show that microthreads in each crosslinking condition and with either EtO or EtOH treatments have an initial toe region of increasing elongation with little change in stress. This region was most visible with UNX microthreads,

where increasing elongation resulted in no increase in stress, while larger increases in stress were apparent in the toe region of crosslinked microthreads. From the comparisons of the stress-strain curves, there were no apparent changes in the mechanical characteristics of the fibrin microthreads for any of the crosslinking conditions, with respect to either EtO or EtOH treatment methods.

Previous studies indicate the mechanical properties of uncrosslinked collagen sponges did not change with EtO sterilization,^[27] while the ultimate tensile strength (UTS) of uncrosslinked silk fibroin scaffolds decreased with EtO sterilization,^[4, 28] demonstrating that changes to the mechanical properties of protein scaffolds are material dependent. Quantitative analyses of the mechanical properties of fibrin microthreads showed that the sterilization process did not significantly change the UTS of the scaffolds, except for two conditions. The UTS values of EtO sterilized UNX microthreads were twofold lower than unsterilized UNX or EtOH disinfected UNX microthreads (Figure 3A) and the UTS values of EtOH disinfected UV40 microthreads were significantly greater than unsterilized UV40 microthreads. Similarly, the EtO or EtOH treatment process did not significantly affect the maximum tangent modulus (MTM) of the fibrin microthreads, except for EtOH disinfected UV40 crosslinked fibrin microthreads, which were significantly stiffer than EtOH disinfected UNX microthreads (Figure 3B). EtO sterilized EDCn microthreads had significantly lower strains at failure (SAF) values than EtOH disinfected or unsterilized EDCn microthreads, and EtOH disinfected EDCa microthreads failed at significantly higher strains than EDCa microthreads that were unsterilized or sterilized using EtO (Figure 3C). EtO sterilized UNX microthreads failed at significantly lower loads than unsterilized UNX controls (Table 1). While EtO sterilization may yield mechanically weaker UNX microthreads, this problem may be mitigated by bundling multiple microthreads together for load bearing applications.

3.2. Ethanol Disinfection Reduces the Wet Diameter and Swelling Ratio of Fibrin Microthreads

To analyze the effects of either EtO or EtOH treatments on the structural organization of fibrin fibrils within the microthreads, swelling ratios were calculated to estimate interfibrillar interactions and water absorption. Sterilization did not significantly change the wet diameters or swelling ratios of most sample groups, but EtOH disinfection significantly decreased the wet diameters and swelling ratios of UNX and UV40 microthreads with respect to both their respective unsterilized or EtO sterilized treatment groups (Figure 4A). EtOH disinfection functions, in part, by dehydrating materials. This is consistent with both the decreases in the diameter and swelling ratio observed in the EtOH disinfected UNX and UV40 microthreads and it may explain the mechanism for the changes in the structural properties of EtOH disinfected microthreads.^[29, 30] Additionally, the swelling ratio of EtOH disinfected EDCa microthreads was significantly lower than unsterilized EDCa microthreads. EtO sterilization significantly increased the swelling ratio of EDCn microthreads with respect to unsterilized or EtOH disinfected EDCn microthreads.

3.3 FT-IR Spectra Demonstrate Chemical Differences between Sterilization Method and Crosslinking Strategies

To characterize how the crosslinking and sterilization procedures affect the surface chemistry of fibrin microthreads, samples of the scaffolds were examined using FT-IR analyses. All spectra showed characteristic fibrin peaks including an N-H stretch ($\sim 3270\text{ cm}^{-1}$), amide I ($\sim 1630\text{ cm}^{-1}$), amide II ($\sim 1520\text{ cm}^{-1}$), and amide III (1240 cm^{-1}) (Figure 5A), similar to current literature.^[31, 32] Two distinct patterns were visible between $1400\text{--}1000\text{ cm}^{-1}$, with differences observed in two regions (Figure 5B). Unsterilized and EtO sterilized UNX and UV microthreads had similar spectra (Pattern 1). All other spectra followed a remarkably similar pattern that was distinct from these four groups (Pattern 2). This included all EDC crosslinked microthreads regardless of sterilization technique, and all EtOH disinfected microthreads (including UV and UNX). The two regions that differed between spectra were identified as $\sim 1170\text{ cm}^{-1}$ (C-O stretch of carboxylic acids) and $\sim 1040\text{ cm}^{-1}$ (C-N stretch of amines in lysine side chains).^[33] Peaks within these regions were absent from pattern 2. Additionally, EtO sterilized EDCa microthreads displayed an increase in the C-H stretch region between $2950\text{--}2910\text{ cm}^{-1}$ and a new peak in the C-H stretch region at 2850 cm^{-1} (Figure 5C).

Differences in the chemical properties of the sterilized fibrin microthreads may be attributed to differences in both the crosslinking conditions and the reactivity of the EtO gas with the crosslinked fibrin materials, which present different amounts of reactive functional groups such as amino and carboxyl groups within the protein structure of the scaffolds.^[6] Aspartic acid and glutamic acid (carboxyl-containing amino acids), as well as lysine (amine-containing amino acid) comprise 12.2%, 13.5%, and 7.8% of fibrinogen, respectively.^[34] Carbodiimide crosslinking depletes many of the free amino and carboxyl groups to form amide bonds, and free amine groups are further depleted in EDCa microthreads compared to EDCn microthreads.^[10] This reaction was consistent with the results of the FT-IR analyses, which showed that all EDC crosslinked microthreads followed pattern 2, suggesting that there was a reduction in the amount of carboxylic acids and amines.

The toxicity of ethylene oxide is attributed to its alkylation of nucleophilic sites on DNA.^[35] However, the reactivity of EtO is not restricted to DNA, and has been shown to alkylate proteins at similar binding sites.^[35, 36] An increase in C-H stretch bands were observed for the EDCa microthreads sterilized with EtO, which may suggest that alkylation occurred on these scaffold materials. The increased number of available amine and carboxyl functional groups in uncrosslinked microthreads may have also been affected by EtO exposure, resulting in reduced interchain interactions, and ultimately the observed reduction in UTS of UNX microthreads. Additionally, EtO sterilized EDCn microthreads failed at lower strain values, and had increased swelling ratios compared with unsterilized or EtOH disinfected EDCn microthreads, supporting the hypothesis that some functional groups remain within the structure of EDCn microthreads that may be affected by EtO exposure.

To evaluate the relative amounts of amide bonds present between sample groups, the ratio between amide I ($\sim 1630\text{ cm}^{-1}$) and C-H stretch ($\sim 2955\text{ cm}^{-1}$) (AM1:CH), as well as the ratio between amide II ($\sim 1520\text{ cm}^{-1}$) and C-H stretch ($\sim 2955\text{ cm}^{-1}$) (AM2:CH) were investigated (Figure 5D). EDCa microthreads had significantly larger AM1:CH ratios than

UNX and UV microthreads, independent of sterilization treatment. EDCn and EDCa microthreads had significantly larger AM2:CH ratios than UV microthreads and had qualitatively larger AM2:CH ratios than UNX microthreads, independent of sterilization treatment. These data further support the hypothesis that the number of amide bonds increases with EDC treatment.

When disinfected with EtOH, UNX and UV microthreads showed very similar spectra to EDCn and EDCa crosslinked microthreads. Ethanol is often used as an additive in the sedimentation of proteins from aqueous solution as it has a low dielectric constant relative to water, which can result in an increase in the Coulomb force of attraction between oppositely charged groups. Ethanol has also been shown to induce conformational changes in nucleic acid structures, which, due to diminished repulsive forces, can result in nucleic acid aggregation.^[37, 38] Similarly, the EDC crosslinking mechanism initiates the linkage of adjacent amine and carboxyl side groups on the surfaces of macromolecular constructs such as fibrin, increasing the intermolecular interactions within the construct. As such, a possible explanation for the similarities between the FT-IR spectra of EDC crosslinked and EtOH disinfected microthreads is that the Coulomb attraction between the carboxyl and amine side groups of UNX and UV microthreads generated amide bonds in a similar mechanism as EDC crosslinked microthreads.

3.4. Ethylene Oxide Gas Reduces Cell Attachment to Microthreads

To determine how sterilization affected cell attachment to the scaffold surface, EtOH or EtO sterilized microthreads were seeded with C2C12 myoblasts. No contamination was observed at any time point with either sterilization method. It is important to note that while ethanol effectively kills most pathogens, it does not have sporicidal ability.^[7, 8] Therefore, while our data suggest that ethanol was an effective disinfectant for *in vitro* analysis of fibrin microthreads by inhibiting microbial or fungal growth in antibiotic and antimycotic free medium, this method of disinfection is not sufficient for clinical applications. Cell attachment at 1 day was measured by quantifying the cell density along the surface of the microthread bundles using Hoechst nuclear dye. Hydrated, sterilized UNX microthreads compacted into a structure that approximated a large, single microthread (Figure 6A and B). Ethanol disinfected UV40 and EDCn microthreads also compacted into structures that approximated a large, single thread, however the topography of each discrete microthread remained visible within the superstructure of the microthread bundle (Figure 6C and E). In contrast, EDCa microthreads remained discrete, individual microthreads (Figure 6G). Bundles of EtO sterilized EDCn microthreads also compacted into structures that approximated a large, single thread while retaining the topography of discrete microthreads (Figure 6F), but bundles of UV40 and EDCa microthreads both remained discrete, individual microthreads (Figure 6D and H). There was a qualitative decrease in cell attachment on EtOH disinfected UV40 microthreads after seeding (Figure 6C and D), and an increase in cell density on EtOH disinfected EDCn and EDCa microthreads, although these differences were not statistically significant ($p=0.06$, Figure 7A). The cell density on EtO sterilized EDCa microthreads was significantly lower than the cell density on EtO sterilized EDCn or UNX microthreads. EtO sterilized EDCa microthreads had significantly lower cell

densities than EtOH disinfected EDCa microthreads, and the seeding density of other microthread treatment groups was not affected by the sterilization method.

To estimate the ability of the differently crosslinked materials to support cell growth, cell density was also quantified on microthread bundles 3 days after seeding. The surface of all EtOH disinfected microthread bundles appeared to be confluent (Figure 8A, C, E, and G). The surface of EtO sterilized UNX, UV40, and EDCn microthreads were also confluent (Figure 8B, D, and F), while EtO sterilized EDCa microthreads appeared to be subconfluent (Figure 8H). There was a significant decrease in the cell density of EtO sterilized EDCa microthreads compared to all other similarly sterilized microthreads (Figure 7B). Additionally, EtO sterilized EDCa microthreads had significantly lower cell densities than EtOH disinfected EDCa microthreads, while all other microthread groups had similar cell densities regardless of the sterilization method.

It is interesting to note that in this study, cell attachment decreased on EtO sterilized EDCa microthreads while myoblast proliferation was unaffected regardless of sterilization technique. Previously, seeding density was shown to significantly increase on EtOH disinfected EDCa microthreads.^[10] While we do not report significant increases in this study, there is a qualitative increase with respect to UNX microthreads. Myoblast nuclei that attached to EtO sterilized EDCa microthreads exhibited normal, healthy morphologies, suggesting that the surfaces of these sterilized microthreads were not cytotoxic. Further, the differences in cell density between EtOH disinfected and EtO sterilized EDCa microthreads were proportionally the same between 1 and 3 days of culture, suggesting that this sterilization method did not affect cell viability over time. These data further suggest that EtO sterilization alkylated the surface of EDCa microthreads to facilitate a decrease in cell attachment, but did not affect proliferation. In fact, the proliferation of myoblasts did not appear to be altered by the sterilization method, despite the decrease in cell attachment observed with EtO sterilized EDCa microthreads.

Ethylene oxide sterilization is completed through alkylation which may result in additional C-H bonds.^[39] The C-H stretch anticipated for this increase would be in the 2950-2850 cm^{-1} region of the spectra. We observed the broadening of one peak and the addition of a second peak within this region of the spectrum of EtO sterilized EDCa microthreads compared to all other spectra. EtO reacts most readily with carboxylic acids,^[40] and it is possible that primary amines were hydrolyzed into carboxyl groups while in acidic buffer, which may explain why the EtO sterilized EDCa microthreads were the only group to display an increase in C-H stretch sites. This increase in alkylation may either cause steric inhibition, or the masking of cell binding sites on the surface of the microthreads, resulting in significantly reduced attachment of myoblasts on EtO sterilized EDCa microthreads.

4. CONCLUSIONS

Sterilization of biopolymer scaffolds is a critical step in the use of these materials as constructs for the development of engineered tissues. An ongoing challenge in the sterilization of biopolymer-based scaffolds is implementing a strategy that does not adversely affect the structural or mechanical properties of the constructs.^[6, 27] The goal of

this study was to analyze how two treatment methods, ethylene oxide (EtO) sterilization and ethanol (EtOH) disinfection, affected the structural properties of both uncrosslinked and crosslinked fibrin microthreads. These sterilization methods are commonly used in laboratory settings, and EtO has been increasingly used in commercial settings.^[6] In this study, we show that terminal sterilization of fibrin microthreads with EtO or EtOH has a limited effect on their mechanical, structural, and biochemical properties. Ethanol disinfection reduced the diameter and swelling ratio of fibrin microthreads, suggesting that it dehydrates and compacts the fibrin fibrils within the microthread structure. Our FT-IR data supports the hypothesis that EtO sterilization alkylates proteins in UNX and EDCa microthreads, significantly decreasing the tensile strength of UNX microthreads and the initial attachment of myoblasts on EDCa microthreads. The mechanical strength and chemical properties of EDCn microthreads appear to be unaffected by either sterilization method. Ethylene oxide (EtO) sterilization and ethanol (EtOH) disinfection were equally effective in preventing microbial or fungal growth for three days in antibiotic and antimycotic free medium. Together, these analyses suggest that sterilization techniques may alter the functional properties of fibrin-based scaffolds such as fibrin microthreads. Ultimately, the findings from these studies will enable the strategic design of fibrin-based scaffolds for facile translation from benchtop to clinical application.

Acknowledgments

This research was funded in part by US Army (W81XWH-11-1-0631), NIH R01-HL115282 (G.D.P.), and NIH F31-DE023281 (J.M.G.). The authors wish to thank Rebecca Sharpe and Megan Read for their assistance in the collection of the mechanical data.

References

1. Stoppel WL, White JC, Horava SD, Henry AC, Roberts SC, Bhatia SR. J Biomed Mater Res B Appl Biomater. 2014; 102:877. [PubMed: 24259507]
2. Freytes DO, Stoner RM, Badylak SF. J Biomed Mater Res B Appl Biomater. 2008; 84:408. [PubMed: 17618508]
3. Ahmed M, Punshon G, Darbyshire A, Seifalian AM. J Biomed Mater Res B Appl Biomater. 2013; 101:1182. [PubMed: 24039066]
4. de Moraes MA, Weska RF, Beppu MM. J Biomed Mater Res B Appl Biomater. 2014; 102:869. [PubMed: 24259492]
5. Delgado LM, Pandit A, Zeugolis DI. Expert review of medical devices. 2014; 11:305. [PubMed: 24654928]
6. Mendes GC, Brandao TR, Silva CL. American journal of infection control. 2007; 35:574. [PubMed: 17980234]
7. Holy CE, Cheng C, Davies JE, Shoichet MS. Biomaterials. 2001; 22:25. [PubMed: 11085380]
8. Best M, Springthorpe VS, Sattar SA. American journal of infection control. 1994; 22:152. [PubMed: 7943926]
9. Ratner, BD. Biomaterials science : an introduction to materials in medicine. 3. Elsevier/Academic Press; Amsterdam; Boston: 2013. p. liii
10. Grasman JM, Page RL, Dominko T, Pins GD. Acta Biomater. 2012; 8:4020. [PubMed: 22824528]
11. Page RL, Malcuit C, Vilner L, Vojtic I, Shaw S, Hedblom E, Hu J, Pins GD, Rolle MW, Dominko T. Tissue Eng Part A. 2011; 17:2629. [PubMed: 21699414]
12. Dennis RG, Smith B, Philp A, Donnelly K, Baar K. 2008
13. Huang YC, Dennis RG, Larkin L, Baar K. Journal of Applied Physiology. 2005; 98:706. [PubMed: 15475606]

14. Grassl ED, Oegema TR, Tranquillo RT. J Biomed Mater Res A. 2003; 66:550. [PubMed: 12918038]
15. Huynh TN, Tranquillo RT. Ann Biomed Eng. 2010; 38:2226. [PubMed: 20431952]
16. Twardowski RL, Black LD 3rd. Ann Biomed Eng. 2014; 42:1074. [PubMed: 24435656]
17. Morin KT, Dries-Devlin JL, Tranquillo RT. Tissue Eng Part A. 2014; 20:553. [PubMed: 24083839]
18. Robinson PS, Tranquillo RT. Tissue Eng Part A. 2009; 15:2763. [PubMed: 19368523]
19. Kural MH, Billiar KL. Biomaterials. 2014; 35:1128. [PubMed: 24210873]
20. Wendel JS, Ye L, Zhang P, Tranquillo RT, Zhang JJ. Tissue Eng Part A. 2014; 20:1325. [PubMed: 24295499]
21. Sakiyama-Elbert SE, Hubbell JA. J Control Release. 2000; 69:149. [PubMed: 11018553]
22. Wood MD, Gordon T, Kemp SWP, Liu EH, Kim H, Shoichet MS, Borschel GH. Biotechnol Bioeng. 2013; 110:1272. [PubMed: 23239194]
23. Mosesson MW. J Thromb Haemost. 2005; 3:1894. [PubMed: 16102057]
24. Grasman JM, Pumphrey LM, Dunphy M, Perez-Rogers J, Pins GD. Acta Biomater. 2014; 10:4367. [PubMed: 24954911]
25. Cornwell KG, Pins GD. J Biomed Mater Res A. 2007; 82:104. [PubMed: 17269139]
26. Proulx MK, Carey SP, Ditroia LM, Jones CM, Fakharzadeh M, Guyette JP, Clement AL, Orr RG, Rolle MW, Pins GD, Gaudette GR. J Biomed Mater Res A. 2011; 96:301. [PubMed: 21171149]
27. Olde Damink LH, Dijkstra PJ, Van Luyn MJ, Van Wachem PB, Nieuwenhuis P, Feijen J. J Biomed Mater Res. 1995; 29:149. [PubMed: 7738061]
28. Hofmann S, Stok KS, Kohler T, Meinel AJ, Muller R. Acta biomaterialia. 2014; 10:308. [PubMed: 24013025]
29. Siritientong T, Srichana T, Aramwit P. AAPS Pharm Sci Tech. 2011; 12:771.
30. Braghirolli DI, Steffens D, Quintiliano K, Acasigua GA, Gamba D, Fleck RA, Petzhold CL, Pranke P. J Biomed Mater Res B Appl Biomater. 2014; 102:700. [PubMed: 24259451]
31. Devi MP, Sekar M, Chamundeswari M, Moorthy A, Krithiga G, Murugan NS, Sastry TP. B Mater Sci. 2012; 35:1157.
32. Perumcherry SR, Chennazhi KP, Nair SV, Menon D, Afeesh R. Tissue engineering Part C, Methods. 2011; 17:1121. [PubMed: 21902615]
33. Silverstein, RM.; Bassler, GC.; Morrill, TC. Spectrometric identification of organic compounds. 5. Wiley; New York: 1991. p. x
34. Triantaphyllopoulos E, Triantaphyllopoulos DC. The Biochemical journal. 1967; 105:393. [PubMed: 6056635]
35. Dellarco VL, Generoso WM, Sega GA, Fowle JR 3rd, Jacobson-Kram D. Environmental and molecular mutagenesis. 1990; 16:85. [PubMed: 2209569]
36. Ross WC. Advances in cancer research. 1953; 1:397. [PubMed: 13057709]
37. Eickbush TH, Moudrianakis EN. Cell. 1978; 13:295. [PubMed: 203402]
38. Piskur J, Rupprecht A. FEBS letters. 1995; 375:174. [PubMed: 7498493]
39. Ehrenberg L, Hiesche KD, Osterman-Golkar S, Wenneberg I. Mutation research. 1974; 24:83. [PubMed: 4851925]
40. Hugo, WB. Inhibition and destruction of the microbial cell. Academic Press; London, New York: 1971. p. xiii

Figure 1. Overview illustrating (A) the setup for uniaxial tensile testing of fibrin microthreads. (B) Microthreads were mounted onto vellum frames with a precut window of 2.0 cm, (C) mounted onto the uniaxial testing machine and the edges of the vellum frame were cut, and (D) were uniaxially loaded until failure.

Figure 2.

Characteristic stress-strain curves for (A) uncrosslinked (UNX), (B) UV crosslinked (UV40), (C) EDC crosslinked in neutral buffer (EDCn), or (D) EDC crosslinked in acidic buffer (EDCa) fibrin microthreads after terminal sterilization with ethylene oxide gas (EtO) or 70% ethanol (EtOH) disinfection. All microthreads displayed elastic toe regions, and in the case of carbodiimide crosslinked microthreads, was followed by a rapid increase in stress until failure.

Figure 3.

Mechanical properties of crosslinked fibrin microthreads as a function of sterilization method. Microthreads were fabricated, crosslinked, and terminally sterilized with ethylene oxide gas (EtO) or disinfected with 70% ethanol (EtOH). Microthreads were subsequently loaded to failure under uniaxial tension and the (A) ultimate tensile strength (UTS), (B) maximum tangent modulus (MTM), and (C) strain at failure (SAF) were calculated. Brackets indicate statistical significance between corresponding groups and * indicates statistical significance with the remaining treatment groups in the same crosslinking condition using one-way ANOVA with Holm-Sidak post hoc analysis ($p < 0.05$, $n = 24$).

Figure 4.

Structural properties of fibrin microthreads as a function of sterilization method. (A) Plot showing changes of wet diameters, and (B) changes in the swelling ratios of fibrin microthreads terminally treated with either 70% ethanol (EtOH) or ethylene oxide gas (EtO). Brackets indicate statistical significance between corresponding groups and * indicates statistical significance with the remaining treatment groups in the same crosslinking condition using one-way ANOVA with Holm-Sidak post hoc analysis ($p < 0.05$, $n = 24$).

Figure 5. FT-IR analysis of control, ethanol (EtOH) disinfected, or ethylene oxide (EtO) sterilized fibrin microthreads that were not crosslinked (UNX), UV crosslinked (UV), or carbodiimide crosslinked in acidic (EDCa) or neutral (EDCn) buffers. (A) Characteristic spectra are depicted with regions of interest highlighted (B and C). (D) Normalized amide quantities were compared using one-way ANOVA with Holm-Sidak post hoc analysis. Brackets indicate statistical significance between corresponding groups ($p < 0.05$).

Figure 6. Representative images of attachment of C2C12 myoblasts on bundles of 10 crosslinked microthreads treated with 70% ethanol (EtOH, A, C, E, and G) or ethylene oxide gas (EtO, B, D, F, and H). Ethanol disinfected UNX, UV40, and EDCn microthreads compacted into a structure approximating a large, single thread, while EtO sterilization only facilitated the compaction of UNX and EDCn microthread bundles into what approximates as a single, large thread. Scale bar = 100 μ m.

Figure 7.

Density of C2C12 myoblasts seeded on crosslinked fibrin microthreads treated with either 70% ethanol (EtOH) or ethylene oxide (EtO) for (A) 1 day or (B) 3 days. Brackets indicate statistical significance between corresponding groups using one-way ANOVA with Holm-Sidak post hoc analysis and † indicates statistical significance between sterilization methods in the same crosslinking condition via Student's t-test ($p < 0.05$, $n = 24$).

Figure 8.

Representative images of C2C12 myoblasts on bundles of 10 crosslinked microthreads treated with 70% ethanol (EtOH, A, C, E, and G) or ethylene oxide gas (EtO, B, D, F, and H) after 3 days of culture. The surface of all EtOH disinfected microthread bundles appear to be confluent, while the surface of EtO sterilized EDCa microthreads appear to be subconfluent (H). Scale bar = 100 μm .

The mechanical and structural properties of uncrosslinked and crosslinked fibrin microthreads after ethylene oxide (EtO) sterilization or ethanol (EtOH) disinfection.

Table 1

	Sample Size	Diameter (μm)			Swelling Ratio	UTS (MPa)	MTM (MPa)	SAF (mm/mm)	Load (mN)
		Dry	Wet						
UNX	Unsterilized	26	47 $\pm$ 10	110 $\pm$ 18	2.4 $\pm$ 0.4	2.3 $\pm$ 1.0	22.3 $\pm$ 15.1	0.322 $\pm$ 0.139	22 $\pm$ 14
	EtOH	26	47 $\pm$ 13	99 $\pm$ 27 [*]	2.1 $\pm$ 0.3 [*]	2.5 $\pm$ 1.1	29.9 $\pm$ 24.8	0.310 $\pm$ 0.128	17 $\pm$ 7
	EtO	26	51 $\pm$ 9	124 $\pm$ 20	2.4 $\pm$ 0.3	1.3 $\pm$ 0.5 [*]	18.0 $\pm$ 17.3	0.324 $\pm$ 0.131	15 $\pm$ 5 ^a
	Unsterilized	30	53 $\pm$ 8	89 $\pm$ 17	1.7 $\pm$ 0.3	4.0 $\pm$ 1.2	71.4 $\pm$ 55.5	0.168 $\pm$ 0.083	24 $\pm$ 8
UV40	EtOH	30	53 $\pm$ 12	70 $\pm$ 18 [*]	1.4 $\pm$ 0.3 [*]	8.7 $\pm$ 6.0 ^a	152.9 $\pm$ 105.1 [*]	0.133 $\pm$ 0.075	29 $\pm$ 16
	EtO	41	54 $\pm$ 9	83 $\pm$ 18	1.5 $\pm$ 0.2	6.9 $\pm$ 7.7	103.1 $\pm$ 83.0	0.146 $\pm$ 0.056	30 $\pm$ 16
	Unsterilized	32	49 $\pm$ 8	81 $\pm$ 20	1.6 $\pm$ 0.3	10.8 $\pm$ 8.2	139.6 $\pm$ 80.0	0.198 $\pm$ 0.085	48 $\pm$ 20
EDCn	EtOH	35	46 $\pm$ 12	75 $\pm$ 24	1.6 $\pm$ 0.3	11.1 $\pm$ 7.6	147.4 $\pm$ 108.3	0.209 $\pm$ 0.075	50 $\pm$ 28
	EtO	34	48 $\pm$ 12	86 $\pm$ 17	1.8 $\pm$ 0.3 [*]	8.5 $\pm$ 4.3	151.7 $\pm$ 107.0	0.143 $\pm$ 0.103 [*]	40 $\pm$ 21
	Unsterilized	24	50 $\pm$ 12	80 $\pm$ 13	1.7 $\pm$ 0.3	6.8 $\pm$ 3.8	158.0 $\pm$ 88.7	0.069 $\pm$ 0.053	33 $\pm$ 17
EDCa	EtOH	29	55 $\pm$ 15	77 $\pm$ 24	1.4 $\pm$ 0.3 ^a	10.0 $\pm$ 6.8	154.6 $\pm$ 101.0	0.149 $\pm$ 0.071 [*]	37 $\pm$ 21
	EtO	24	51 $\pm$ 13	78 $\pm$ 25	1.6 $\pm$ 0.4	10.9 $\pm$ 7.4	219.2 $\pm$ 190.5	0.102 $\pm$ 0.082	47 $\pm$ 43

Data is presented as mean $\pm$ standard deviation.

^a indicates statistical significance with unsterilized microthreads within the same crosslinking condition and

^{*} indicates statistical significance with the remaining treatment groups in the same crosslinking condition using one-way ANOVA with Holm-Sidak post hoc analysis ($p < 0.05$).