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2015

Peracetic Acid: A Practical Agent for Sterilizing Heat-Labile Polymeric Tissue-engineering Scaffolds

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Peracetic Acid: A Practical Agent for Sterilizing Heat-Labile Polymeric Tissue-engineering Scaffolds

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Dentistry at Virginia Commonwealth University.

By

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April 14, 2015

Acknowledgment

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POLYMERIC TISSUE-ENGINEERING SCAFFOLDS

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Major Director: Parthasarathy A. Madurantakam, Assistant Professor, Philips Institute, School of Dentistry

Abstract: Advanced biomaterials and sophisticated processing technologies aim to fabricate

tissue-engineering scaffolds that can predictably interact within a biological environment at a

cellular level. Sterilization of such scaffolds is at the core of patient safety and is an important

regulatory issue that needs to be addressed prior to clinical translation. In addition, it is crucial

that meticulously engineered micro- and nano- structures are preserved after sterilization.

Conventional sterilization methods involving heat, steam and radiation are not compatible with

engineered polymeric systems because of scaffold degradation and loss of architecture. Using

electrospun scaffolds made from polycaprolactone (PCL), a low melting polymer, and

employing spores of *Bacillus atrophaeus* as biological indicators, we compared ethylene oxide,

autoclaving and 80% ethanol to a known chemical sterilant, peracetic acid (PAA), for their ability to sterilize as well as their effects on scaffold properties. PAA diluted in 20% ethanol to 1000 ppm or above, sterilized electrospun scaffolds in 15 min at room temperature while maintaining nano-architecture and mechanical properties. Scaffolds treated with PAA at 5000 ppm were rendered hydrophilic, with contact angles reduced to zero degrees. Therefore, PAA can provide economical, rapid and effective sterilization of heat-sensitive polymeric electrospun scaffolds used in tissue-engineering.

Keywords: Peracetic acid, sterilization, polymeric scaffolds, electrospinning, *Bacillus atrophaeus* spores.

INTRODUCTION

Tissue-engineering is a rapidly evolving field that aims to develop functional tissue substitutes by integrating advanced engineering principles and improved understanding of cell behavior. The ultimate goal of tissue-engineering and regenerative medicine is to improve the quality of life in patients by promoting true regeneration of structure and function of tissue compromised by disease or surgery [1]. Scaffold-based tissue-engineering is a popular strategy that involves the seeding and culture of specific cell types in an environment that mimic the native extracellular matrix (ECM). The ideal ECM analogs are engineered to be 3D instructional matrices that possess the physical, chemical and biological cues to promote tissue repair and regeneration [2]

Metals and alloys, ceramics and polymers, either alone or in combination have been traditionally used to rehabilitate patients with failing or removed organs. While metals and ceramics are inherently strong and may possess favorable mechanical properties for orthopedic applications, they are designed to be non-degradable and possess limited processability. Polymers are being increasingly used in tissue-engineering because they are biocompatible, can be rendered biodegradable (by imparting appropriate chemistry), do not elicit host immune reactions (unlike natural polymers) and can be mass produced with little batch-to-batch variability. In addition, their composition, structure, mechanical properties and degradation rates can be tailored to suit specific needs [3]. Polycaprolactone (PCL) is a synthetic polymer, placed

on the FDA's generally regarded as safe (GRAS) list, exhibits excellent biocompatibility,

complete degradation *in vivo* and has been approved for drug delivery and medical devices

applications. PCL has been successfully used as micro- and nano- spheres in controlled drug

delivery systems [4-6]; in sutures as a co-polymer with glycolide (Monacryl® by Ethicon); as a

root canal filler [7]; and in tissue-engineering applications [8, 9].

Among different techniques available to generate 3D porous polymeric scaffolds,

electrospinning is a versatile technique that consistently reproduces the sub-micron fibrous

morphology of the native ECM. The process involves dissolving a biodegradable polymer in an

organic solvent at high concentrations and subjecting this viscous solution to high voltage (tens

of kilovolts). At a critical voltage, the electrostatic charge overcomes the surface tension of the

polymer drop and polymer chains entangle to form a stable jet. As the charged jet travels towards

a grounded target under the influence of electric field, the solvent evaporates and the fibers are

collected as dry, fibrous, non-woven mats. The scaffold composition, fiber diameters and

alignment can be readily controlled by the operator to tailor tissue-specific properties [10].

Tissue engineered products are devices intended to be in direct contact with living tissue

and are regulated by the FDA for safety and efficacy. One of the fundamental requirements for

such a device is the need to be completely sterile (and not merely disinfected) prior to

implantation. The Center for Disease Control defines sterilization as a process that destroys or

eliminates all forms of microbial life while disinfection describes a process that eliminates many

or all pathogenic microorganisms, except bacterial spores, on inanimate objects [11]. Hence it is

imperative that an appropriate sterilization method is chosen to ensure safety as well as maintain

material properties and preserve engineered micro- and nano-scaled features of polymeric

scaffolds. Current sterilization processes employed by the health care industry, including autoclave, gamma irradiation and ethylene oxide, cannot be readily applied to tissue engineered products because of the biomaterial involved i.e. synthetic polymers. Polyesters are the most widely used class of polymers because of their biocompatibility and biodegradability. However, being hydrolytically unstable, they cannot be subjected to moist heat (autoclaving); high energy gamma radiation degrades polymeric backbone, reduce molecular weight and alter degradation profiles [12]. Ethylene oxide (EtO) alters scaffold properties by penetrating into polymeric networks and reacting with chemical groups [13].

Given the translational nature of tissue-engineering research and constant innovation in polymer systems and their processing technologies, the issue of sterilization needs to be periodically revisited. In this study, we systematically explore the feasibility of using peracetic acid (PAA) as a chemical sterilant for polymeric tissue-engineered scaffolds and compared it with two accepted methods of sterilization (EtO and autoclaving) and a high-level disinfectant (80% ethanol). We included the latter because of its widespread use in tissue engineering studies. We chose a widely used polymer, PCL, to represent polymers with low melting points and electrospun it to produce porous 3D scaffolds with defined nano-topography. Our aim was to identify the process conditions (concentration, contact time and temperature) necessary to achieve sterility while maintaining scaffold integrity. Since spores are routinely used as biological indicators for sterilization [14], we inoculated electrospun scaffolds with spores of *Bacillus atrophaeus* and exposed them to different sterilization treatments. To our knowledge, this is the first study to evaluate the effectiveness of sterilization using spores as biological indicators in the context of electrospun polymeric scaffolds. In addition to assaying spore

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survival, the effects of sterilization processes on scaffold properties including fiber morphology,

permeability, hydrophilicity and tensile modulus were evaluated.

MATERIALS AND METHODS

2.1. Electrospinning: PCL (Sigma, MW 80,000, melting point 59-64°C) was dissolved in 1,1,1,3,3,3-hexafluro-2- isopropanol (HFP, Oakwood Products, SC) at a concentration of 100 mg/ml. Electrospinning apparatus (EC-DIG, IME Technologies, Netherlands) was used to generate nanofibers. Process conditions were optimized (rate: 7 ml/h, air-gap distance: 12.5 cm, applied needle voltage: +25 kV) to generate continuous non-woven fibers that were collected onto a rotating cylindrical drum mandrel (100 mm diameter at 1000 rpm). After electrospinning, scaffolds were removed from the mandrel, dried in a fume hood for 30 min and stored in an airtight desiccator until use. Electrospun PCL (e-PCL) scaffolds were cut using dermal biopsy punches (Acuderm, FL) for use in experiments.

2.2. Characterization of *B. atrophaeus* Spores: *B. atrophaeus* spores (ATCC #9372) were purchased as suspensions in 20% ethanol (10^8/ml) from Moog Medical Devices Group, NY and stored at 4°C. A total of 10^6 spores were diluted in 1 ml of de-ionized water (DI water) and two subsequent (1:50) serial dilutions were plated on TSA (tryptic soy-agar) plates using the EddyJet2 Spiral Plating System (NeuTec Group, NY). The plates were then transferred to the incubator at 35°C and checked for colonies after 18 h.

2.3. Sterilization Efficacy of Peracetic Acid (PAA): PAA was purchased as a 39% solution (Sigma) in acetic acid and hydrogen peroxide. Different concentrations of PAA (100, 500, 1000, 2500 and 5000 ppm) were obtained by diluting the stock (390,000 ppm) in appropriate volume of

DI water. Initial experiments to identify the minimal effective concentration were done by directly exposing the spore suspensions to different concentrations of PAA for 5 minutes at room temperature, plating these solutions on solid agar and evaluating colonies, as described in 2.2. Absence of colonies is a more important parameter while assessing terminal sterilization since their evidence represents a failure to achieve sterility. Thus, the actual numbers of colonies are irrelevant and were not recorded. For experiments involving scaffolds, a second diluent was introduced; in addition to DI water, the PAA was also diluted in 20% ethanol. This is because we observed significantly better wetting of spore solution (in 20% ethanol) on scaffolds than DI water.

2.4. Scaffold Inoculation with *B. atrophaeus* Spores and Culture: Our goal was to inoculate 10^6 spores onto each 10 mm disc of e-PCL fabric. We observed poor loading of spores onto scaffolds when used as dilute solution (as described in 2.2) and hence chose to inoculate without dilution (10 µl spore suspension). Circular discs, placed into 24-well microplate, were inoculated with 10^6 spores and allowed to dry for 30 min. These spore-laden scaffolds were then subjected to different methods of sterilization (described in section 2.6). At the end of defined sterilization cycle, scaffolds were transferred into 5 ml of tryptic soy broth (TSB) and cultured in a mechanical shaker for 3 days at 35^oC. Turbidity of the broth indicated bacterial growth and was interpreted as an indicator of inadequate sterilization.

2.5. Scanning Electron Microscopy: Air-dried electrospun scaffolds (before and after various sterilization protocols) were mounted on aluminum stubs using standard double-sided tape, sputter coated with gold and examined at an accelerating voltage of 20 kV using JEOL JSM

5610LV scanning electron microscope. Average fiber diameters were calculated from a total of 50 randomly selected fibers from corresponding SEM images using Image J (NIH).

2.6. Sterilization Treatment and Efficacy Testing of Electrospun Scaffolds: Dry, spore-laden PCL scaffolds were subjected to 6 different sterilization regimens: ethylene oxide (EtO); autoclaving; 80% ethanol; 1000 ppm, 2500 ppm and 5000 ppm of peracetic acid (PAA). EtO sterilization was carried out at 50°C for 16 h (including aeration time), while autoclaving was performed at 121°C at 15 psi for 15 min. Scaffolds for these treatments were placed in self-sealing pouches (Henry-Schein) containing appropriate chemical indicators to verify that conditions for sterilization were met. Scaffolds for ethanol treatment were immersed in an 80% solution (in DI water) for 30 min and rinsed three times with PBS for 10 min per wash. Stock solution of PAA was diluted in either DI water or 20% ethanol solution (in DI water) to prepare different concentrations and PCL scaffolds incubated for 15 min at room temperature on an orbital shaker. The contact times was increased from 5 minutes (section 2.3) to account for 3D nature of the scaffold and allow adequate time for PAA to infiltrate the porous network and come in contact with the spores. Scaffolds treated with each sterilization regimen, as well as untreated controls, were then transferred into 5 ml of tryptic soy broth (TSB) and cultured in a mechanical shaker for 3 days at 35°C. Again, an increase in culture turbidity, due to bacterial growth, was used as an indicator of inadequate sterilization.

2.7. Contact Angle Measurements: Changes in surface properties of e-PCL scaffolds (treated and controls) were determined by measuring the contact angle using a Rame´-Hart 200 contact-angle goniometer. A sessile drop (2-4 µl volume) of DI water was placed on the surface of the scaffold using a micro-syringe and allowed to equilibrate for a period of 10 sec. The image of the

drop was captured and analyzed using DROPImage (Rame´-Hart Instrument, NJ) for contact angle measurements. A total of 6 readings were performed for each scaffold type.

2.8. Scaffold Permeability: A modification of flow meter developed in our laboratory and described previously [15] was used to calculate scaffold permeability. Instead of a steady hydrostatic pressure (provided by an elevated reservoir) and gravity-assisted flow, we adapted a micro-filtration assembly (EMD Millipore Corporation, MA) and employed suction to provide the driving force for filtration. Electrospun scaffolds were cut into 25 mm circular discs and their thickness recorded using a micrometer (Mitutoyo America Corporation, IL). Scaffolds were placed on top of a Type 316 stainless steel screen (100 mesh, filtration area of 2.1 cm^2), edges sealed using clear PTFE gaskets and the attachment secured to a 300 ml borosilicate glass funnel using an anodized aluminum clamp. The apparatus was attached to a vacuum pump that generated a suction of 25 inches mercury (corresponds to approximately 4.9 inches mercury of positive pressure). The funnel was filled with 300 ml DI water and the time required for 50 ml to flow through the membrane was recorded. Scaffold permeability was calculated from an average of 4 readings (for each scaffold type) and used in Darcy's equation ($\tau = Q\eta h_s/Ftp$), where, τ represents scaffold permeability in darcy units (d), Q is the fluid volume passed through the scaffold in time t, η is the viscosity (0.89 cp for water at 25^oC), h_s is scaffold thickness, F is the filtration area and p is the applied pressure.

2.9. Uniaxial Tensile Testing: Uniaxial tensile testing was performed according to our previous published studies [16] . Briefly, scaffolds from each group (n=6) were punched into 'dog-bones' (2.75 mm wide at their narrowest point with a gage length of 7.5 mm) and tested on an MTS Bionix 200 testing system with a 50 N load cell (MTS Systems Corp.) at an extension rate of

10.0 mm.min^{-1} . Elastic modulus, strain at break and energy to break were calculated and recorded by MTS TestWorks 4.0.

2.10. Stability of Peracetic Acid: PAA was prepared in different concentrations from 100-2000 ppm in DI water as well as 20% ethanol solution and stored air-tight at room temperature for up to 3 weeks. The solutions were subsequently tested for PAA concentration every 3 d using colorimetric MQuant™ test strips specific for peracetic acid and sensitive in 100-2000 mg/L (ppm) range (EMD Millipore, Germany). Manufacturers' instructions were followed to test PAA concentrations and any changes over time were recorded.

2.11. Statistical Analysis: Values were presented as means and standard deviation where appropriate. The scaffold types were compared using ANOVA and significant differences were described using Tukey's HSD. All analyses were performed using SAS software (JMP version 10, SAS Institute Inc., Cary NC).

RESULTS

3.1. Electrospun Scaffold and Spore Characterization: Porous, nanofibrous scaffolds were generated following optimization of electrospinning conditions. SEM analyses revealed the average fiber diameter was 0.92±0.52 µm. There was a broad distribution of fibers with fiber diameters ranging 136 nm to 2100 nm. SEM of spores showed typical rod-shaped structure, with the smaller dimension less than 1 µm, size was small enough to penetrate into the depths of porous fibrous matrix (**Figure 1**). Spores loaded onto scaffolds could not be visualized even at high concentrations, possibly due to the porous nature of electrospun scaffolds as well as lack of color contrast.

3.2. *B. atrophaeus* Spore Culture and Sensitivity to PAA: Untreated spores promptly germinated on the surface of TSB agar to form discrete reddish-orange colonies within 18 h (**Figure 2**). Longer incubation times led to coalescence and difficulty distinguishing individual colonies. Exposure of spore suspensions to PAA (diluted in DI water) resulted in marked reductions in colony forming units. The number of colonies decreased significantly at 100 ppm (visual) but isolated colonies could still be seen at 500 ppm. However, no colonies were found at 1000 ppm or above. **Figure 3** is representative of the results obtained with 3 trials. Hence, we established that 1000 ppm was the minimal sporicidal concentration of PAA at room temperature.

3.3. Effects of Sterilization:

For each of the sterilization treatments on e-PCL scaffolds, we validated the sterilization process using the spores of *B. atrophaeus* as a biological indicator. In addition, we investigated the effects of the process on the physical and mechanical properties of the scaffolds. The results are discussed in the same order.

3.3.1. Sterilization Efficacy:

Both EtO and autoclaving are established methods of sterilization and expectedly destroyed all spores. Scaffolds treated with 80% ethanol demonstrated heavy bacterial loads similar to untreated controls. This is not surprising given that 80% ethanol is a high-level disinfectant incapable of killing spores and hence is not a viable option for terminal sterilization. Since 1000 ppm was identified to be the minimal sporicidal concentration of PAA, lower concentrations (100 and 500 ppm) were ignored and assays on e-PCL scaffolds were performed with 1000, 2500 and 5000 ppm only. Spore-inoculated scaffolds, challenged to different concentrations of PAA diluted in DI water, showed incomplete sterilization even at 1000 and 2500 ppm (data not shown). Lack of efficacy at these sporicidal concentrations was attributed to inadequate wetting of PCL scaffold and resultant decreased access of PAA to spores within the scaffold. In order to improve the wetting characteristics of hydrophobic polymer scaffold, PAA was diluted in 20% ethanol, the same solution in which the spores were originally suspended. This modification dramatically improved the efficacy of PAA demonstrated by complete sterilization at 1000 ppm and above, consistent with our earlier observation with spore suspensions (**Figure 4**).

3.3.2: Effects of sterilization methods on physical and mechanical properties:

3.3.2a. Gross Morphology and SEM: EtO treated electrospun scaffolds showed minimal gross dimensional change, but the scaffolds became translucent and brittle. Autoclaving induced massive melting and coalescence of polymer and completely destroyed the integrity of the scaffold. Further, scaffolds subjected to EtO and autoclaving showed complete loss of fibrous architecture and fusion of independent fibers under SEM (**Figure 5**). Scaffolds treated with chemical sterilants (80% ethanol and different concentrations of PAA) did not show any appreciable change in either macroscopic (photographic imaging) or microscopic (SEM) scale compared to controls.

The scanning electron micrographs of scaffolds treated with PAA diluted in DI water and 20% ethanol are shown in **Figure 6**. The fibrous morphology of the scaffolds was significantly altered by treatment with PAA diluted in DI water in a concentration-dependent manner; individual fibers started to fuse into bundles with evidence of fiber breakage at higher concentrations. PAA diluted in 20% ethanol showed a tendency towards thinning of fibers but preserved open porous architecture even at 5000 ppm. Statistical analyses confirmed significant effect of PAA concentration on fiber diameter depending upon the diluent ($p < 0.001$). Scaffolds treated with PAA diluted in DI water showed a significant difference in fiber diameter ($p < 0.001$); fiber diameters at 2500 ppm and 5000 ppm were larger than all other concentrations but were not different from one another (2500 ppm mean = 2.03±1.02 μm vs. 5000 ppm mean = 1.82±0.81 μm). Concentration-dependent effects on fiber diameter were not observed in scaffolds treated with PAA diluted in 20% ethanol ($p > 0.8$).

3.3.2b. Scaffold Hydrophilicity: Contact angle measurements following different sterilization treatments were analyzed to indicate hydrophilicity or wettability of the scaffolds. Generally,

surfaces are termed hydrophilic when the water contact angle is less than 90° and hydrophobic, if contact angle is more than 90°. **Figure 7** shows representative image of an actual drop placed on differently treated surfaces. Untreated control PCL scaffolds are highly hydrophobic (contact angle around 120°); EtO, autoclaving and 80% ethanol treatments make them hydrophilic as seen by reduced contact angles. Scaffolds treated with PAA at 1000 and 2500 ppm, in either diluent, did not significantly alter the wetting properties. However, at 5000 ppm, there was a dramatic decrease in the contact angles. **Figure 8** is quantitative representation of average of contact angles measured from 6 replicates for each scaffold type. Scaffolds treated with PAA at 5000 ppm diluted in DI water decreased contact angles by more than half, whereas PAA in 20% ethanol completely soaked up the water and brought the contact angle to zero.

3.3.2c. Scaffold Permeability: The permeability of the PCL scaffold to water was highest in the control untreated PCL scaffold and decreased with increasing concentrations of PAA until the effect plateaued off at 2500 ppm (**Figure 9**, $p<.001$). The permeability of scaffolds treated with PAA at 2500 ppm was not significantly different than 5000 ppm, nor was it different than when using 80% ethanol. This correlates well with the observation on scaffold hydrophilicity; a hydrophilic scaffold is expected to interact with water and decrease the flow rate. Scaffolds treated with PAA diluted in DI water demonstrated high variations in permeability, due to heterogeneity in wetting characteristics (data not shown).

3.3.2d. Mechanical Properties: Since PAA diluted in DI water were not sporicidal at high concentrations, induced unfavorable changes in fiber morphology and produced inconsistent data for scaffold permeability, we did not perform mechanical testing on these samples. The results of mechanical testing of scaffolds treated with 80% ethanol and different PAA concentrations are

shown in **Figure 10.** EtO and autoclaved samples could not be mechanically tested because of loss of scaffold integrity. It is interesting to note that the modulus was not affected by the concentration of PAA used ($p > 0.06$). Values for energy to break and strain at break indicate a tendency towards brittleness with increasing PAA concentrations up to 2500 ppm (statistically not significant). However, at 5000 ppm, the scaffolds were not statistically different from controls for the same properties ($p = 0.007$ and $p = 0.010$ respectively).

3.4. PAA Stability: PAA is at equilibrium with acetic acid and hydrogen peroxide and is particularly unstable at low concentrations [17]. PAA at 100 ppm started degrading around 7 days as determined by visual comparison with manufacturer- provided shade guide. Higher concentrations of PAA (>200 ppm) did not show any degradation for a period of 3 weeks when stored air-tight at room temperature. In addition, stability of PAA was not affected by the diluent used. Hence, PAA at concentrations necessary for sterilization (> 1000 ppm) could be prepared in large volumes and stored for a minimum of three weeks.

DISCUSSION

The aim of tissue-engineering is to develop viable functional alternatives for failing tissues and organs. However, the strategies pursued have evolved from purely cell- or biomolecule- based approaches to the current paradigm of scaffold-based tissue-engineering. This involves seeding and culturing specific cell types in engineered 3D matrices designed to simulate the ECM [2]. Such matrices are expected to present appropriate physical, biological and biochemical cues to predictably influence cell behavior [18, 19]. Synthetic polymers are widely used in tissue-engineering because they are biocompatible, biodegradable and can be tailored to possess a wide range of properties. The growing list of polymers [3, 20] and emerging scaffold fabrication technologies [21], provide matrices with a variety of internal architecture and mechanical properties.

Intended to be in direct contact with living tissues, these scaffolds must be terminally sterilized prior to implantation. Product sterility cannot be assumed even if fabricated in a 'clean room' because the machinery and starting materials are not sterile. Moreover, normally benign bacteria can become pathogenic when present on the surface of devices [22]. These factors make scaffold sterilization a vital issue to be resolved prior to clinical translation. Synthetic polymers used in tissue engineered scaffolds possess low melting points, are susceptible to hydrolysis and possess intricate architecture at micro-or nano- scale, all of which can be affected by the sterilization process. Mechanical and surface properties, toxicity and biocompatibility of

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scaffolds can all be potentially altered by sterilization. Hence careful evaluation of scaffolds before and after sterilization is required to identify a sterilization method that is benign to the polymer, device and the patient [22]. In this context it is important to realize that standard sterilization practices (including autoclaving, ethylene oxide and use of high energy irradiation) are not specifically suited for polymeric systems employed in tissue-engineering.

Autoclaving with pressurized moist steam at 120°C for 15 min is not a viable option for sterilizing polymers with low melting points. Further, most biocompatible polymers are hydrolytically unstable and exposure to moisture can accelerate degradation, reduce shelf life and alter mechanical properties [23]. EtO Is a reactive gas that can penetrate into polymeric networks, react with their chemical groups, cause polymer degradation and alter scaffold dimensions [24]. In addition, EtO is carcinogenic and needs to be extensively degassed over many hours prior to packaging [13]. High energy irradiation is an efficient sterilization method that may preserve the morphology of 3D scaffolds, but it dramatically decreases the polymer molecular weight and hence, accelerates degradation [11].

Limitations of conventional modes of sterilization in tissue-engineering have led researchers to explore alternatives, especially in the past few years. Shearer *et al.* [25] found that PAA and antibiotic solutions were effective in sterilizing hollow fiber and flat sheets of poly (lactide: glycolide) but induced unfavorable changes in morphology but not mechanical properties. Rainer *et al.* compared the effects of different sterilization techniques (ethanol, dry heat, autoclave, UV and plasma treatment) on morphology and crystallinity of electrospun poly-l-lactide scaffolds [26]. Dry heat and autoclave treatments resulted in an increase in crystallinity while low temperature UV and hydrogen peroxide plasma preserved the structural properties.

Siritientong *et al.* [27] evaluated the effects of sterilization on lyophilized sericin-polyvinyl alcohol scaffolds and concluded that gamma irradiation was the most appropriate method even though it degraded the scaffolds by almost 70% in 24 h. These early studies were critically demonstrated that sterilization of tissue engineering scaffolds is as much about maintenance of material properties and architecture as it is about killing microbes. Sterilization assumes greater significance in scaffolds containing proteins or biologics where the risk of denaturation is real and can result in decreased or loss of vital biological activity.

However, a common limitation in aforementioned studies included a lack of uniform model organism tested; many did not specify the source or the identity of the contaminating bacteria. In cases where known bacteria were used, there is no consistency in the choice of the bacterial species. In addition, some groups used unsterilized material as control which can vary widely in their bacterial load or bio-burden. This makes comparison of sterilization methods in the context of tissue-engineering scaffolds difficult.

In contrast, we adopted a standardized format to test sterilization of electrospun scaffolds in terms of microbe identity and number. Spores of *B. atrophaeus* were appropriately chosen as the model organism, considering their routine use (as biological indicators) to validate sterilization processes. We ensured a consistency in bio-burden by inoculating 10 mm discs of e-PCL scaffolds with 10^6 *B. atrophaeus* spores. We employed EtO and autoclaving as positive controls of sterilization and 80% ethanol because of widespread use in tissue engineering studies. We then systematically identified the process conditions for effective sterilization using PAA, a known chemical sterilant at room temperature. Varying the concentration, contact times, we were able to demonstrate that e-PCL scaffolds can be effectively sterilized using PAA without

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subjecting them to harsh processes. This is especially important in the context of tissue-engineering scaffolds because sterilization provides a higher standard of care as well as highest margin of safety for patients, compared with high-level disinfection. We reasoned that spores being the most resistant form of life [14] and present in large numbers on scaffolds, a negative spore test would indicate complete elimination of bio-burden [11] and a sterile scaffold.

The use of chemical agents to reduce bacterial load in polymeric scaffolds is attractive because it allows processing at low temperatures and short duration. Peracetic acid has long been in use as a chemical sterilizing agent because of its strong oxidizing properties. It is available as an equilibrium mixture of acetic acid and hydrogen peroxide and has been extensively used in the food industry because of its high potency and low residual toxicity. PAA denatures proteins, disrupts cell wall permeability and is effective against all known microbes (including spores) even in the presence of organic matter [17, 28]. PAA is effective at low concentrations, low temperatures and reduced contact times compared with traditional methods. PAA is also economical, degrades into non-toxic end products (water, oxygen and carbon dioxide) and can be safely disposed of without affecting the environment [11].

The efficacy of PAA is affected by concentration, contact time, pH and temperature. A commercially available system (Steris Corporation, OH) employs 35% PAA diluted to 200 ppm in water (pH 6.4), at 50-56°C for 23 min. This automated system has been approved for sterilizing medical, surgical and dental instruments including those made from heat-sensitive materials [29]. The STERIS system is optimized for sterilization at near neutral pH to reduce the tendency of PAA to corrode metals; hence the use of PAA at low concentrations (200 ppm).

We needed to significantly deviate from the FDA approved STERIS protocol to account for differences in materials being processed. First, since polymeric scaffolds are sensitive to heat (PCL degraded with EtO exposure at 50°C), our priority was to perform sterilization at room temperature. Second, in contrast to traditional solid surfaces (tubes and instruments), engineered scaffolds are three dimensional, nanofibrous and porous structures. The enormous surface to volume ratio offered by electrospun scaffolds is a huge advantage in tissue-engineering but also presents an opportunity for colonization and survival of spores/ bacteria in the depths of the scaffold. Third, the hydrophobicity of the polymer coupled with highly porous structure makes the scaffold difficult to be wet by PAA. It is known that the device should be completely immersed and all surfaces must be in direct contact with PAA for effective sterilization.

Having established that 1000 ppm PAA killed spores in suspension within 5 min at room temperature, we extended the contact time to 15 min in experiments with scaffolds, taking into account their 3D porous structure. We also found that wettability of the scaffold affected the ability of PAA to kill spores. Since our model polymer (PCL) was hydrophobic, diluting PAA in water (as has been done in STERIS) yielded incomplete sterilization. However, use of 20% ethanol (in DI water) as diluent for PAA significantly improved the wetting characteristic of the scaffold and restored the efficacy of PAA at previously established concentrations. This finding reinforces the fact that PAA needs to be in physical contact with the surface to be effective.

PAA at high concentrations (5000 ppm) also induced favorable surface properties on electrospun scaffolds. Control PCL scaffolds were significantly hydrophobic as reflected by very high contact angles. However, when scaffolds were treated with PAA at 5000 ppm, the scaffold surface became markedly hydrophilic. The extent of this effect was dependent on the diluent,

with contact angles dropping to zero when 20% ethanol was used. This was not unexpected because polyester polymers are known to undergo acid- or alkali- mediated hydrolysis resulting in increased hydrophilicity. Since hydrophilicity directly affects scaffold biocompatibility and favorable host response [30, 31], use of PAA offers the dual advantage of sterilization and inducing favorable surface properties.

Since acid-mediated hydrolysis can potentially affect morphological characteristics and mechanical properties of electrospun scaffolds, we sought to evaluate these effects by SEM and tensile testing. We found that individual fibers tended to fuse and scaffolds demonstrated decreased porosity when PAA was diluted in DI water, yet no appreciable change occurred when PAA was diluted in 20% ethanol, even at 5000 ppm. In addition, mechanical properties were not significantly affected by the PAA treatment.

The limitations of the current study include investigating the effects on one polymer type (PCL) processed by one fabrication technique (electrospinning) and hence cannot be generalized. PAA is a potent chemical agent that can sterilize any surface, yet its practicality needs to be ascertained for various polymers processed differently. Conditions for sterilization and effects on scaffolds will vary and need to be optimized for specific systems. Future work will investigate the biological response of PAA-sterilized scaffolds *in vitro* and *in vivo*.

CONCLUSIONS

Our primary goal was identify to systematically evaluate the feasibility of using peracetic acid (PAA) as an alternative to conventional methods to effectively sterilize electrospun PCL scaffolds. We deliberately chose PCL because of its low melting point and electrospun it to confer defined nanoscale features, whose integrity can be followed during processing. We report that PAA at 1000 ppm (diluted in 20% ethanol) for 15 min at room temperature renders the scaffold sterile and at 5000 ppm dramatically alters the hydrophilicity of the scaffold as well. More importantly, these effects are observed while preserving the morphological and mechanical properties of the scaffold.

Novel biomaterials (smart polymers, carbon nanotubes) and fabrication technologies (including electrospinning, solid freeform fabrication, stereolithography and 3D printing) are being introduced at rapid pace to develop tissue-specific scaffolds. Scaffold sterilization, though the last physical step in product processing should be at the forefront of scaffold development to ensure that neither the novel properties of biomaterials nor the nanoscale architecture that are painstakingly built are altered or lost.

The authors confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Figure 1: SEM images of electrospun PCL scaffold and *B.atrophaeus* spores.
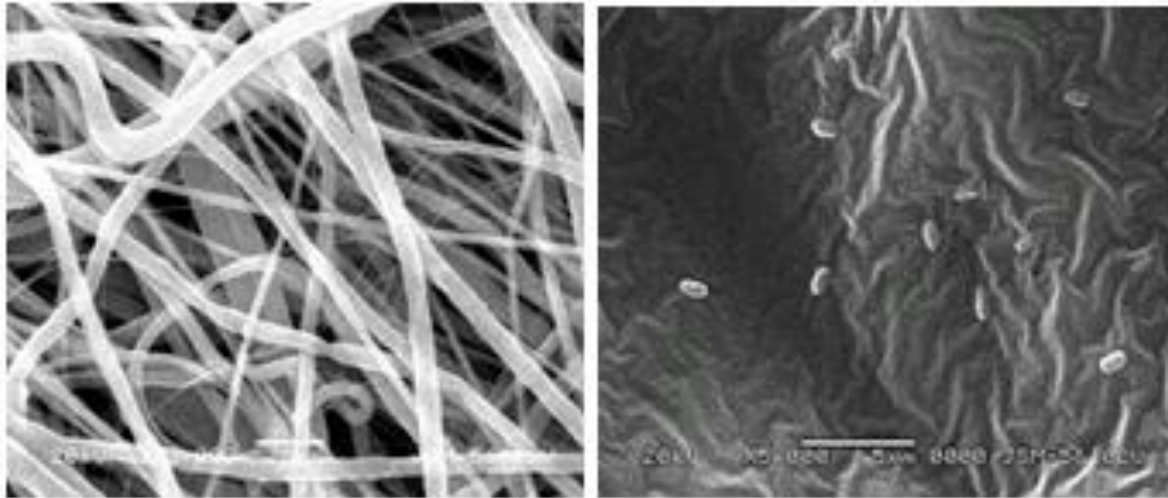
Left: SEM of the e-PCL scaffold demonstrating the fibrous morphology and open porous structure. The mean fiber diameter was 0.92 μm. Right: SEM image of *B. atrophaeus* spores seeded directly on a double sided tape. The rod shaped spores are less than one micron in length. Scale bar is 5 μm in both images.

Figure 2: Optimization of *B. atrophaeus* spore culture.
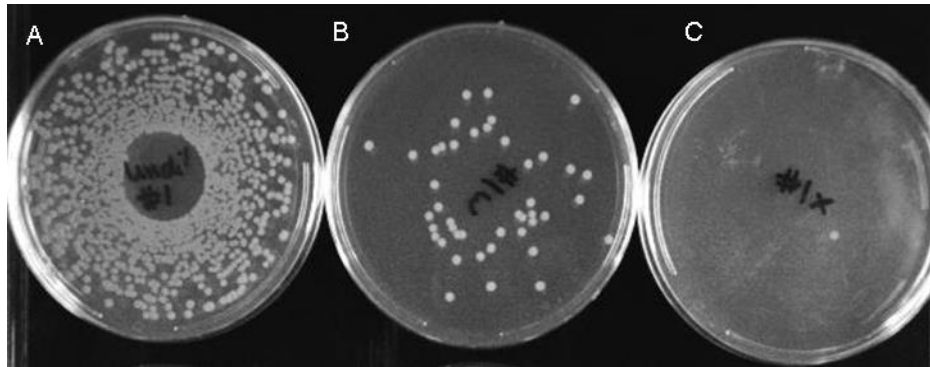
A total of 5 x10^4 spores were seeded onto solid agar plate (A). Subsequent (1:50) serial dilutions are shown in (B) and (C). Discrete reddish-orange colonies were visible upon 18 h incubation at 35°C.

Figure 3: Effect of PAA (diluted in DI water) on spore viability.
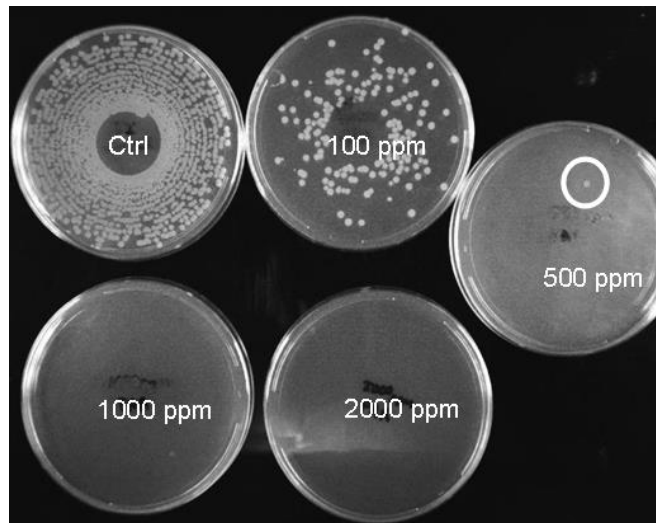
Spores were incubated with different concentrations of PAA for 5 min, suspensions spiral-plated on solid agar and incubated for 18h. Inadequate spore killing was observed at low concentrations (100 and 500 ppm) compared to controls, but complete sterility was seen at 1000 ppm and higher PAA concentrations.

Figure 4: PAA diluted in 20% ethanol effectively sterilizes spore-inoculated PCL scaffolds at 1000 ppm in 15 min at room temperature.
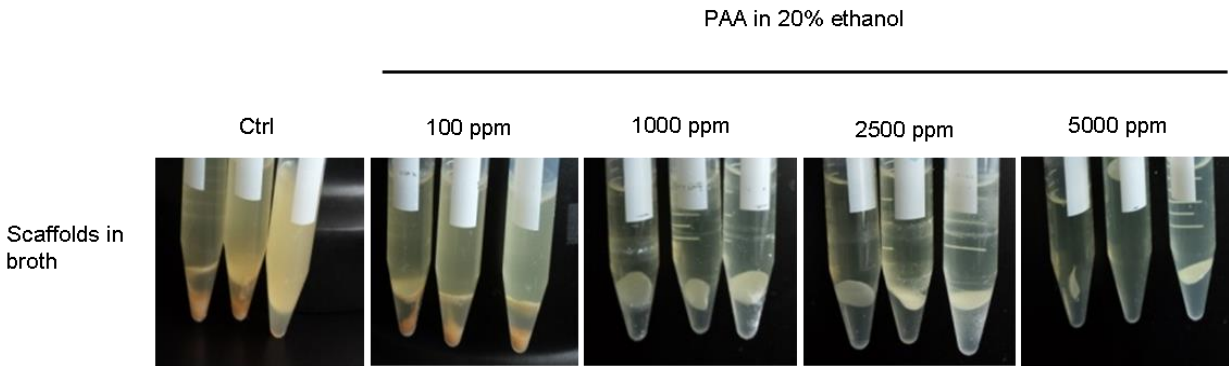
Figure shows scaffolds incubated in broth for 3 days on a mechanical shaker at 35°C. Lack of turbidity in broth for scaffolds treated with higher PAA concentrations (1000 ppm and above) indicates achievement of sterility. Low concentrations (100 ppm) and controls show incomplete spore inactivation and bacterial growth.

Figure 5: Optical and scanning electron micrograph images to illustrate morphology changes of e-PCL scaffolds after sterilization treatments.
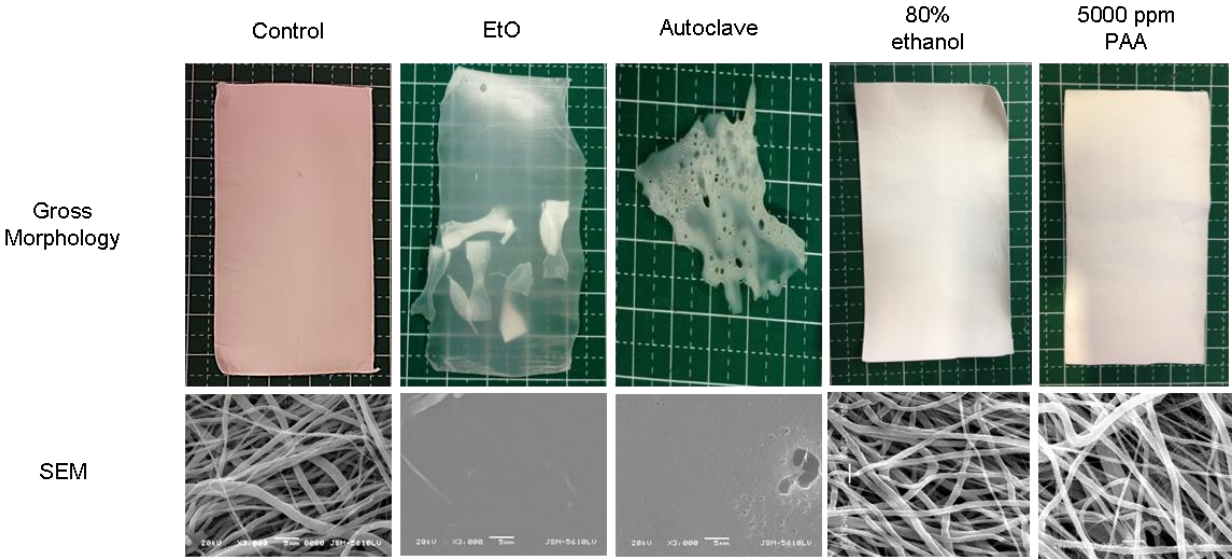

EtO turned scaffolds into a solid, translucent film (dog-bone samples, placed in the same sterilization pouch, fused to the sheet) while autoclaving melted the scaffold. Complete loss of structure fibrous architecture can be observed with both standard sterilization methods. Chemical processing of scaffolds at room temperature (80% ethanol and PAA) did not induce any macro- or microscopic changes in scaffold morphology.

Figure 6: SEM images of e-PCL scaffolds treated with different concentrations of PAA diluted in DI water (top) or 20% ethanol (bottom) for 15 min.
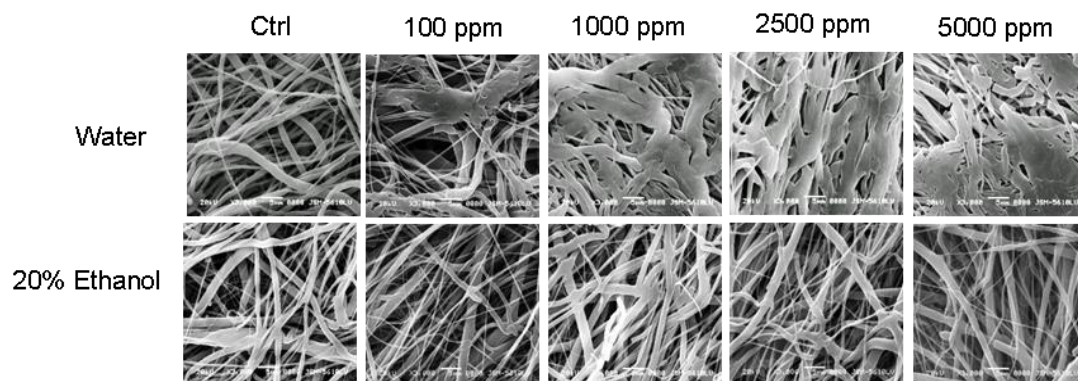

Significant changes in the fibrous structure are seen when DI water was used as a diluent. Such effects were not observed in 20% ethanol group, even at 5000 ppm.

Figure 7: Sessile drop images, after 10 s equilibration, on e-PCL scaffolds subjected to standard sterilization methods (A) and PAA diluted in DI water (B) or 20% ethanol (C).
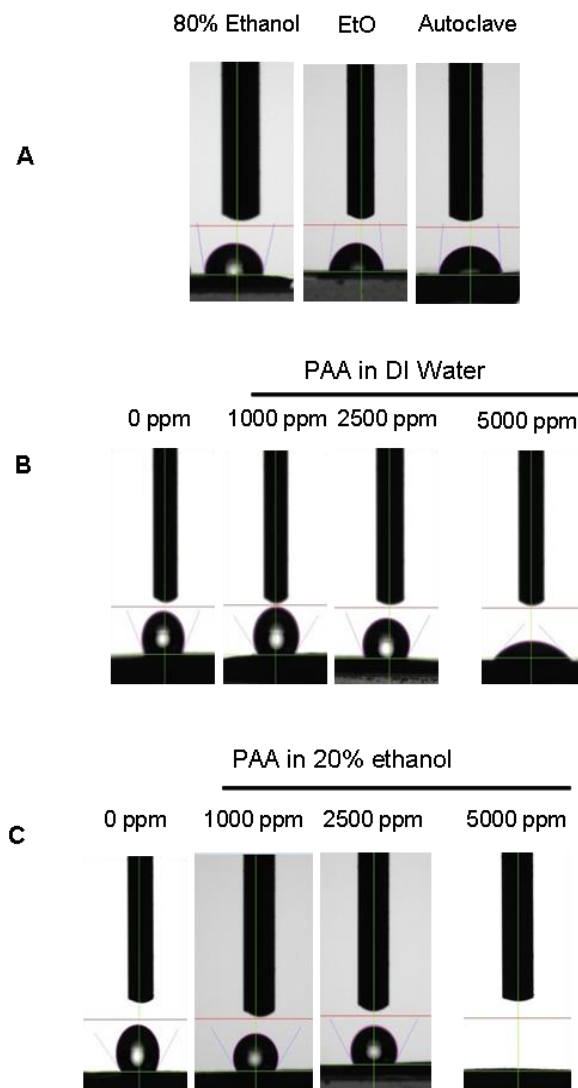

While conventional treatment reduced contact angles appreciably, PAA did not have any significant effect up to 2500 ppm. PAA at 5000 ppm induced a dramatic reduction in contact angle, irrespective of the diluent. The effect was more pronounced when 20% ethanol was used, as seen by complete absorption of the water drop.

Figure 8: Quantification of contact angle measurements of PCL scaffolds from Figure 7.
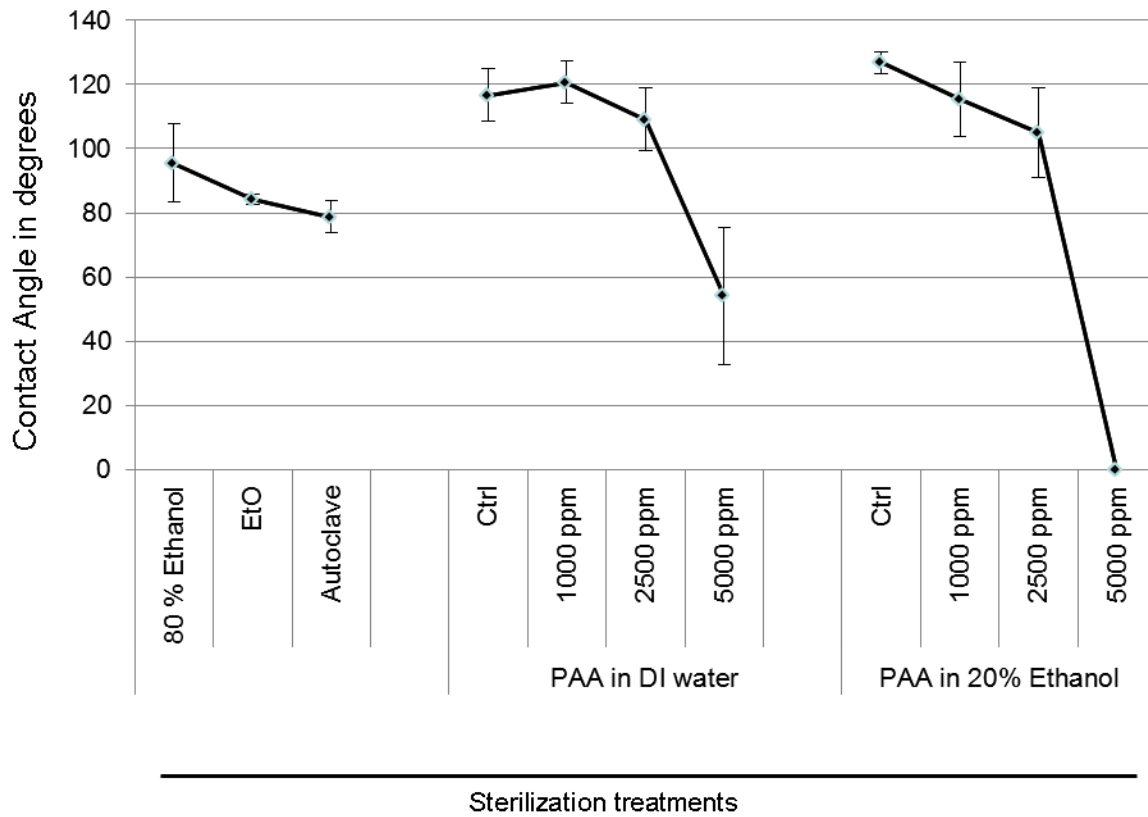

Significant reduction in contact angles were seen following treatment with PAA at 5000 ppm. PAA dilution in DI water reduces the contact angle by half, while dilution in 20% ethanol brought it to zero.

Figure 9: Scaffold permeability (measured in darcy units) determined by flow rate of DI water through treated e-PCL scaffold.
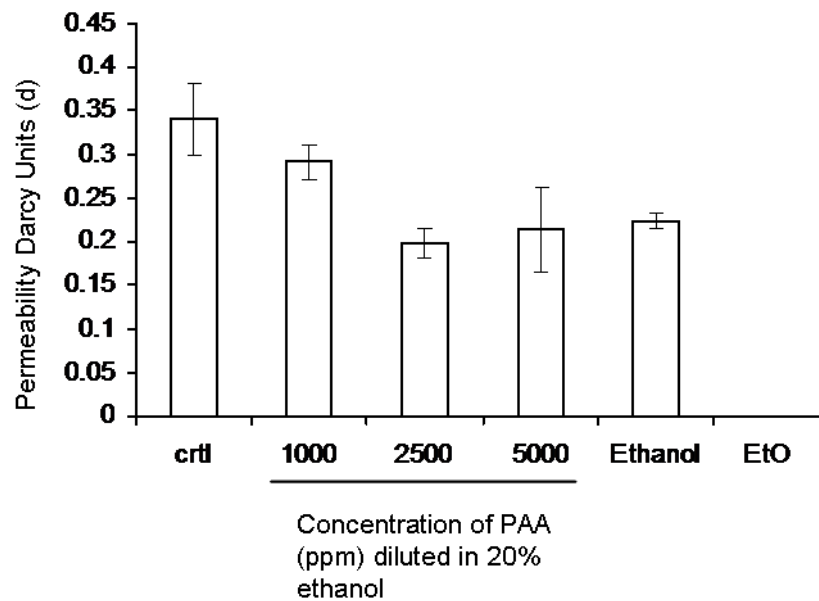
Decreased permeability was observed with increasing concentrations of PAA ($p < 0.001$) while EtO treated scaffolds formed a solid impermeable film.

Figure 10: Mechanical properties of e-PCL scaffolds treated with different concentrations of PAA diluted in 20% ethanol.
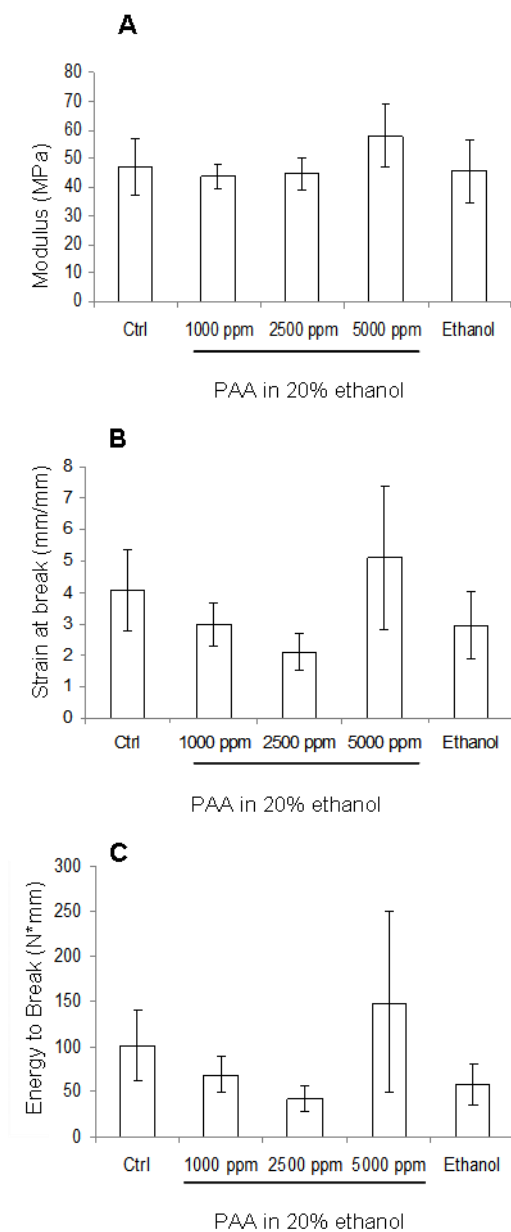
A. Tensile modulus, B. Strain at Break and C. Energy to Break. Control scaffolds refer to scaffolds incubated with 20% ethanol with no PAA. Scaffolds treated with 80% ethanol are also shown. Scaffold properties were not significantly affected by PAA sterilization solution up to a concentration of 5000ppm.