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Peracetic Acid Sterilization of Electrospun Polycaprolactone Scaffolds

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PERACETIC ACID STERILIZATION OF ELECTROSPUN POLYCAPROLACTONE SCAFFOLDS

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

By

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Acknowledgements

As I pen down this piece of bonafide thesis I cannot resist quoting Winston Churchill, “If you’re going through hell, keep going” and thanking many people who have kept me going. I could not have accomplished this without the support and advice from my family, friends and lab mates. I’m dedicating this work to my parents, Yoganarasimha and Sujaya Simha as a tribute to your unconditional love and support. I would like to extend my gratitude to my advisor, Dr. Parthasarathy Madurantakam for his meticulous guidance. It was a blessing to have the opportunity to work with such an amazing researcher. I’m sincerely grateful to my committee members Dr. Christopher Lemmon and Dr. Ning Zhang. I can’t thank you enough Dr. Peter Moon for answering my incessant question on material science and life in general. I especially would like to thank my lab mates Dr. Fadi Hasan and Nick Yesbeck who were always around to provide a helping hand. Their energy, quick smile and frequent encouragement is very much appreciated. Finally for those who are not mentioned by name, you have brought me closer to this day in small and large ways, which is truly valued and reminisced.

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List of Abbreviations

Alv	Polystyrene Scaffolds - Alvetex™
Ctrl-Alv	Alvetex scaffolds disinfected with 70% ethanol
Ctrl-PCL	Electrospun PCL scaffolds disinfected with 70% ethanol
ECM	Extra Cellular Matrix
e-PCL	Electrospun PCL
EtO	Ethylene Oxide
EtOH	Ethanol
HFP	Hexafluoro-2-Propanol
HMDS	Hexamethyldisilazane
MC3T3	Mouse Calvarial Preosteoblast Cell Line
MTS	-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4sulfophenyl)2H-tetrazolium, inner salt
OD	Optical Density
PAA	Peracetic Acid
PAA-Alv	Alvetex scaffolds Sterilized with Peracetic Acid
PAA-Alv+EtOH	Alvetex scaffolds Sterilized with Peracetic Acid & Quenched with Ethanol
PAA-PCL	Electrospun PCL Scaffold Sterilized with Peracetic Acid
PAA-PCL+EtOH	Electrospun PCL Scaffold Sterilized with Peracetic Acid & Quenched with Ethanol
PBS	Phosphate Buffered Saline
PCL	Polycaprolactone
PDO	Polydioxanone
PTFE	Polytetrafluoroethylene
SEM	Scanning Electron Microscope
STS	Sodium Thiosulfate
TCPS	Tissue Culture Plastic
TSA	Tryptic Soy Agar

Abstract

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Sterilization of tissue engineered scaffolds is an important regulatory issue and is at the heart of patient safety. With the introduction of new biomaterials and micro/nano structured scaffolds, it is critical that the mode of sterilization preserve these built-in features. Conventional sterilization methods are not optimal for engineered polymeric systems and hence alternate systems need to be identified and validated. PCL is polyester with a low melting point (heat labile), susceptible to hydrolysis and is popular in tissue engineering. Electrospinning generates some nanoscale features within the scaffold, the integrity of which can be affected by sterilization method. Chapter 1 explores the possibility of using Peracetic acid (PAA) to sterilize polymeric scaffolds that are sensitive to heat or moisture. PAA is a strong oxidizing agent that has been

approved for sterilizing catheters and endoscopes. The ability of PAA to sterilize at room temperature, its breakdown into non-toxic end products and effectiveness at low concentrations are major advantages.

Chapter 2 evaluates the ability of PAA-sterilized PCL scaffolds (PAA-PCL) to support survival and proliferation of mouse calvarial osteoblast cell line, MC3T3. While Ctrl-PCL scaffolds (ethanol-disinfected) showed robust cell survival, PAA-PCL scaffolds induced massive cell death. Following interrelated hypotheses are tested: the observed cytotoxicity was due adsorption of PAA and/or hydrogen peroxide onto PCL fibers during sterilization; and elimination of adsorbed residues will restore scaffold cytocompatibility. Neither extensive aeration nor chemical neutralization with sodium thiosulfate and catalase were effective in improving cell survival. However, quenching PAA-PCL scaffolds in 70% ethanol for 30 minutes effectively removed adsorbed PAA residues and completely restored cell viability and proliferation over a 7 day period. In order to test if PAA-induced toxicity was limited to electrospun PCL scaffolds, commercially available, porous polystyrene scaffolds (Alvetex®) was treated with PAA. Interestingly, a statistically significant increase in cell survival and proliferation resulted with PAA treatment and this was abolished by ethanol quenching. Combined, these results illustrate that PAA treatment can produce diametrically opposite effects on cell survival depending on substrate chemistry and that PAA can be used to effectively sterilize tissue engineering scaffolds without compromising cell viability.

Keywords: peracetic acid, sterilization, electrospinning, polymeric scaffolds, cytotoxicity, desorption, quenching, Alvetex®

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

Chapter 1

1.1 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 diseased/ lost tissue. Scaffold-based tissue engineering is a popular strategy that involves seeding and culture of specific cell types in an environment that mimics the native extracellular matrix (ECM). The ideal ECM analogs are engineered to be 3D instructional matrices that contain all the physical, chemical and biological cues to promote tissue repair and regeneration[1].

Metals and alloys, ceramics and polymers, either alone or in combination have been traditionally used to rehabilitate the patient with missing organs. While metals and ceramics are inherently strong and possess favorable mechanical properties for orthopedic applications, they are non-degradable and possess limited process ability. Polymers are popular materials used in various devices for replacing missing organs or restoring functions of the body because of favorable physical and mechanical properties. Synthetic polymers are being increasingly used in tissue engineering because the composition, structure and hence the properties can be tailored to suit specific needs. In addition to being biocompatible, synthetic polymers can be rendered biodegradable (by imparting appropriate chemistry), does not elicit immune reactions (unlike natural polymers) and can be mass produced with little batch-to-batch variability[2]. Among the synthetic biodegradable polymers, polycaprolactone (PCL) and polydioxanone (PDO) are very popular in the fields of drug delivery, medical devices and tissue engineering because of their excellent biocompatibility, slow yet complete degradation in vivo and its ability to be blended with

other polymers to tailor properties as well as FDA approval. Micro- and nano- spheres of PCL have been used successfully in controlled drug delivery systems[3,4,5]; in sutures as a co-polymer with glycolide (Monacryl® by Ethicon); as a root canal filler[6]; and tissue engineering applications[7,8]. PDO, on the other hand has been extensively used in medical devices as sutures (PDS®, by Ethicon) and tissue engineering applications[9,10,11,12].

Among different scaffold fabrication techniques available to generate 3D porous polymeric scaffolds, electrospinning has been very popular because of its simplicity, versatility and its ability to faithfully reproduce the sub-micron fibrous morphology of the native ECM. The process involves dissolving a biodegradable polymer in a suitable solvent at high concentrations and subjecting this viscous solution to high voltage (typically tens of kilovolts). Under optimal conditions, sufficient polymer chains entanglements occur to form a stable polymer jet that accelerates towards a grounded target driven by the high potential difference. The solvent evaporates during the travel 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[13].

Given the translational nature of tissue engineering research, constant innovation in polymer systems and their processing technologies, the issue of sterilization needs to be periodically revisited., Tissue engineered products are regulated by the FDA for efficacy and safety reasons and as devices intended to be direct contact with living tissue needs to be completely sterile prior to implantation. It is also imperative for the sterilization method to preserve the meticulously engineered micro- and nano-scaled features as well as the material and structural properties of the polymeric scaffolds. Current sterilization processes employed by the health care industry including ethylene oxide, moist steam (autoclave) and gamma irradiation, cannot be readily applied to tissue

engineered products because of the nature of the biomaterial involved i.e. polymers. Since polyester polymers are hydrolytically unstable, subjecting them to autoclaving (moist heat) is not an option; high energy gamma radiation is known to degrade polymeric backbone reducing molecular weight and alter degradation profiles[14]; and ethylene oxide (EtO) has been shown to alter scaffold properties by penetrating into polymeric networks and reacting with chemical groups[15].

This study systematically explore the feasibility of using peracetic acid (PAA) as a chemical sterilant for polymeric tissue-engineered scaffolds, when compared to EtO, autoclave and ethanol. PCL was chosen as the model polymer because of its proven track record and its low melting point. In addition, PCL was subjected to electrospinning to produce porous 3D scaffolds with well-defined nano-topography. The aim was to identify the sterilization conditions (in terms of concentration, contact time and temperature) necessary to achieve complete sterility while maintaining scaffold integrity. Sterilization method was exposed to the highest challenge by inoculating electrospun scaffolds with spores of Bacillus *atrophaeus*. Spores represent the most resistant forms of life and are the gold standard to verify the efficacy of sterilization. The effects of sterilization on scaffold properties including fiber morphology, porosity, permeability, hydrophilicity and tensile modulus was examined. This is the first study to evaluate the biological effectiveness of sterilization against spores in the context of electrospun polymeric scaffolds.

1.2 MATERIALS AND METHODS

1.2.1 Electrospinning: PCL (Sigma, MW 80,000) was dissolved in hexafluroisopropanol (HFP) at a concentration of 100 mg/ml. Electrospinning conditions were optimized (rate: 7 ml/hr, air-gap distance: 12.5 cm, voltage: 25 kV) to generate continuous non-woven nanofibers that were collected onto a rotating cylindrical drum mandrel (1000 rpm). After electrospinning, scaffolds were removed from mandrel, dried in the hood for 30 min and stored in an airtight desiccator until use. Scaffolds were cut using defined punches for use in various experiments.

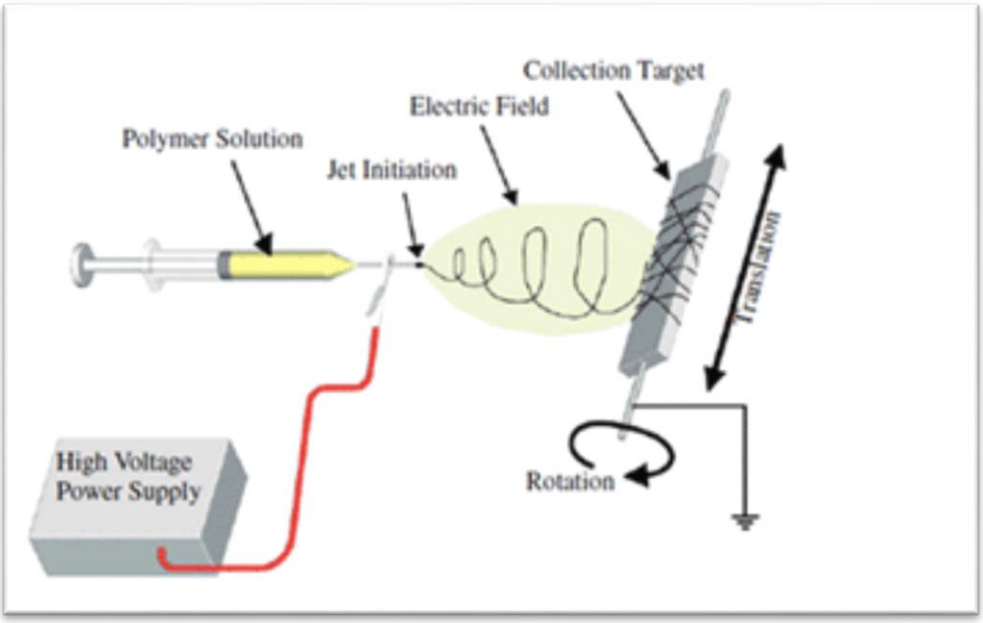

Fig 1: Schematic of Electrospinning.

1.2.2 Characterization of B. *atrophaeus* Spores: B. atrophaeus spores (ATCC #9372) was purchased as suspensions from Moog Medical Devices Group, NY, USA and stored at 4^{o}C. A total of 10^6 spores (10 µl) were added to 990 µl of DI water to be used as a starting concentration for all the experiments. Fifty microliter of this solution as well as two serial dilutions (1:50) was

plated on TSA (tryptic soy-agar) plates using Eddy Jet 2 Spiral Plating System (NeuTec Group, NY). The plates were then transferred to the incubator at 35°C and the colonies were counted using the automated colony counter after 18 hours.

1.2.3 Sterilization efficacy of 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 an appropriate volume of DI water. Initial experiments to identify the minimal effective concentration were performed by directly exposing the spore suspensions to different concentrations of PAA for 5 min at room temperature, plating these solutions on solid agar and evaluating colonies, as described in the section ''Characterization of B. atrophaeus spores''. The absence of colonies is a more important parameter while assessing terminal sterilization, as 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 of the observed significantly better wetting of spore solution (in 20% ethanol) on scaffolds than DI water.

1.2.4 Scaffold Inoculation with B. *atrophaeus* spores and culture: Electrospun PCL fabric was cut into 10 mm discs using a disposable dermal biopsy punch. Discs were placed in the wells of micro-titer plate and intentionally inoculated with 10^6 spores and allowed to dry. After 30 minutes, the scaffolds were subject to different methods of sterilization. Scaffolds were then transferred into 5 ml of Tryptic soy broth and cultured in a mechanical shaker for 3 days maintained at 35°C. Any turbidity in the broth was due to bacterial growth and represented inadequate sterilization.

1.2.5 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 platinum, and examined at an accelerating voltage of 20 kV using a JEOL JSM 5610LV scanning electron microscope (SEM). Average fiber diameters were calculated from a total of 50 randomly selected fibers from corresponding SEM images using Image J (NIH).

1.2.6 Sterilization Treatments: Electrospun scaffolds were subjected to 8 different sterilization regimens: Ethylene oxide (EtO), Autoclave, 80% Ethanol, 100 ppm, 500 ppm, 1000 ppm, 2500 ppm and 5000 ppm of peracetic acid (PAA). The EtO sterilization was done at 50^{o}C for 16 hours while autoclaving was done at 121^{o}C at 15 psi for 15 minutes. Scaffolds were placed in self-sealing pouches (Henry-Schein) containing chemical color indicators for attainment of process parameters. Scaffolds for ethanol treatment were immersed in an 80% solution (in DI water) for 30 minutes and rinsed thrice with PBS for 10 minutes each. PAA was purchased as 39% solution from Sigma which corresponded to 390,000 ppm. The stock PAA was diluted in either DI water or 20% ethanol solution (in DI water) to arrive at the desired concentrations. The scaffolds were incubated for a period of 15 minutes on an orbital shaker unless otherwise stated.

1.2.7 Contact Angle Measurements: The surface characteristic of the electrospun PCL scaffolds (treated and controls) was determined by measuring the contact angle using a Rame´-Hart 200 contact-angle goniometer. A sessile drop (2-4 µl volume) of DI water is placed on the surface of the scaffold using a micro-syringe and allowed to equilibrate for a period of 10 seconds. The image of the drop was captured and analyzed using DROPImage for contact angle measurements. A total of 6 readings were done for every scaffold type.

1.2.8 Scaffold Permeability: Electrospun scaffolds were cut into 25mm discs and thickness recorded using a micrometer. Vacuum filtration assembly was adapted to measure scaffold permeability. The scaffolds were placed on top of a Type 316 stainless steel screen (100 mesh, filtration area of 2.1 cm2). The edges of the scaffolds were sealed using clear PTFE gaskets and

the attachment was secured to a 300 ml funnel using an anodized aluminum clamp. The funnel was filled with DI water and the entire apparatus was attached to a pump that consistently generated a vacuum of 25 inches mercury (corresponding to 4.92 inches of absolute pressure). The DI water was allowed to flow through the membrane after checking for any leaks and the time it took to filter 300 ml was recorded with readings taken after every 50 ml. A total of 4 readings were averaged for each scaffold type to be used in the Darcy's equation to calculate permeability[16].

1.2.9 Uniaxial tensile testing: Uniaxial tensile testing was performed according to previous published studies[17]. Briefly, scaffolds were cut 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 by the MTS software TestWorks 4.0 and recorded. A total of 6 replicates were done for each treatment type.

1.2.10 Stability of Peracetic Acid: PAA was prepared in different concentrations from 100-2000 ppm in DI as well as 20% ethanol solution and stored air-tight at room temperature. The solutions were tested for the PAA concentration every 3 days for 2 weeks 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 quantify the concentration over time and recorded.

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

1.3 RESULTS

1.3.1 Electrospun scaffold and spore characterization: Porous, nanofibrous scaffolds were generated after the optimization of electrospinning conditions. SEM analyses revealed that the average fiber diameter was 0.92 – 0.52 µm. There was a broad distribution of fibers with fiber diameters ranging from 136 to 2100 nm. The SEM of spores showed a typical rod-shaped structure, with the smaller dimension < 1 µm, and the size was small enough to penetrate into the depths of porous fibrous matrix. Spores loaded onto scaffolds could not be visualized even at high concentrations, possibly due to the porous nature of electrospun scaffolds as well as because of the lack of color contrast.

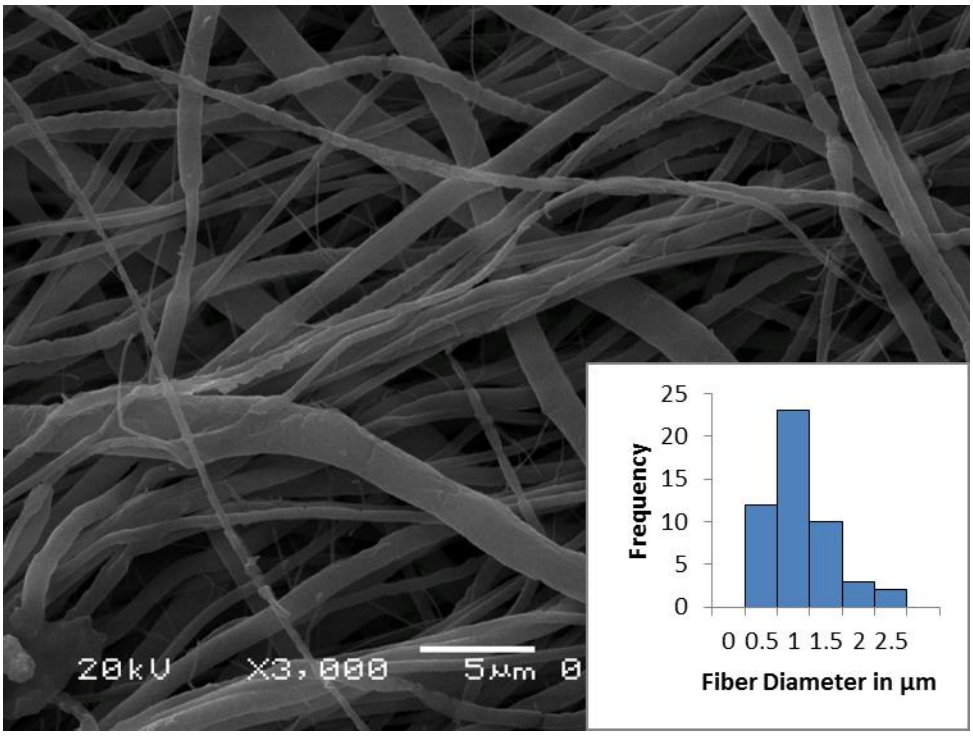

Fig 2: SEM micrograph of electrospun PCL depicting porous, nanofibrous scaffold.

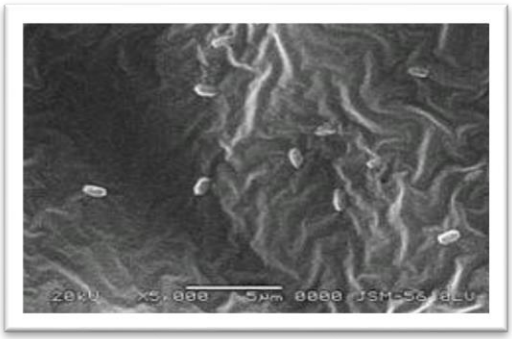

Fig 3: SEM image of B. *atrophaeus* spores.

1.3.2 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. 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 4 is representative of the results obtained with three trials. Hence, we established that 1000 ppm was the minimal sporicidal concentration of PAA at room temperature.

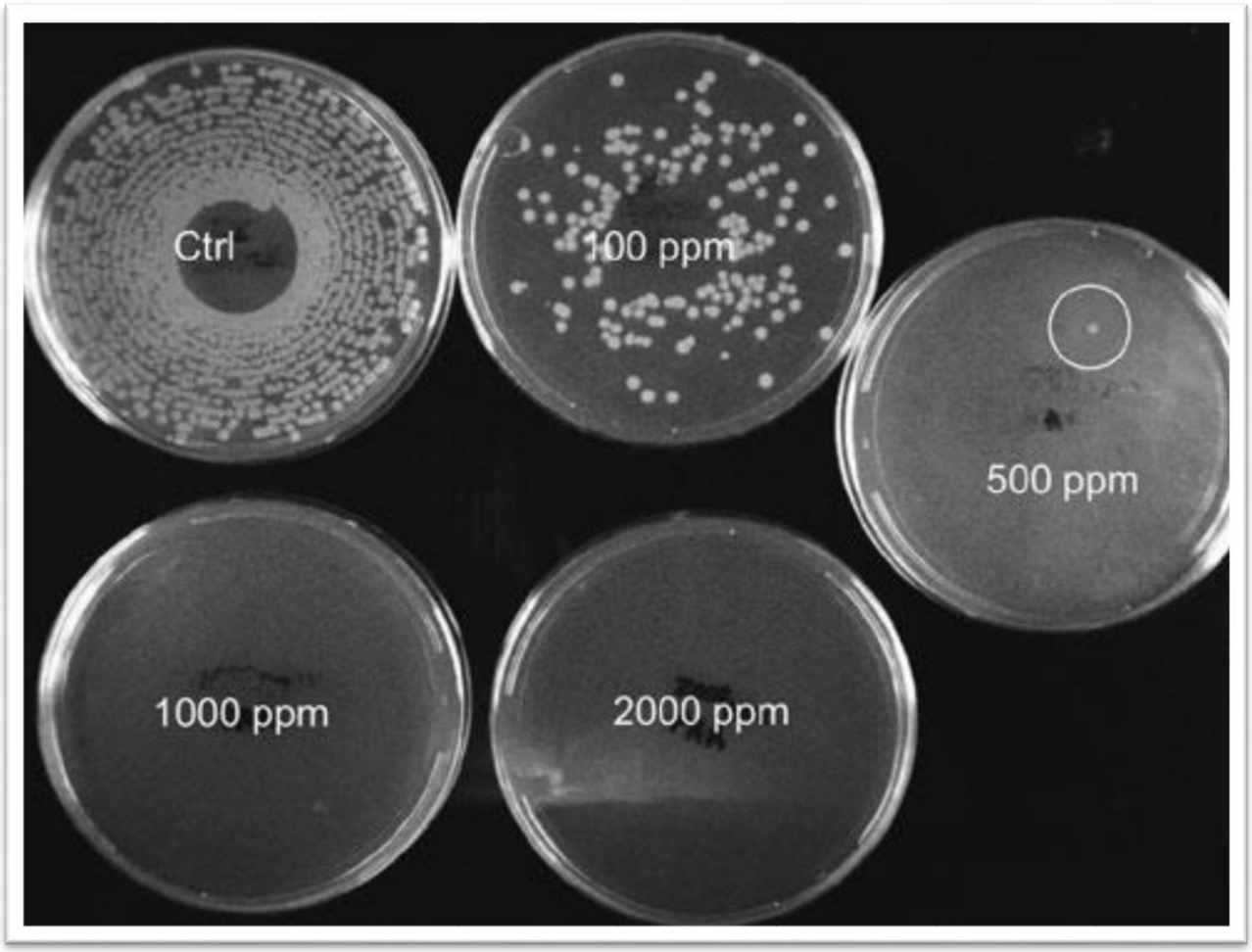

Fig 4: Effect of peracetic acid (PAA) (diluted in deionized water [DI water]) on spore viability. Spores were incubated with different concentrations of PAA for 5 min; suspensions were spiral-plated on solid agar and incubated for 18 h. inadequate spore killing was observed at low concentrations (100 and 500 ppm) compared with controls, but complete sterility was seen at 1000 ppm and higher PAA concentrations.

1.3.3 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.

1.3.3a 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 that is 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 resulted in 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, which was consistent with our earlier observation with spore suspensions (Figure 5).

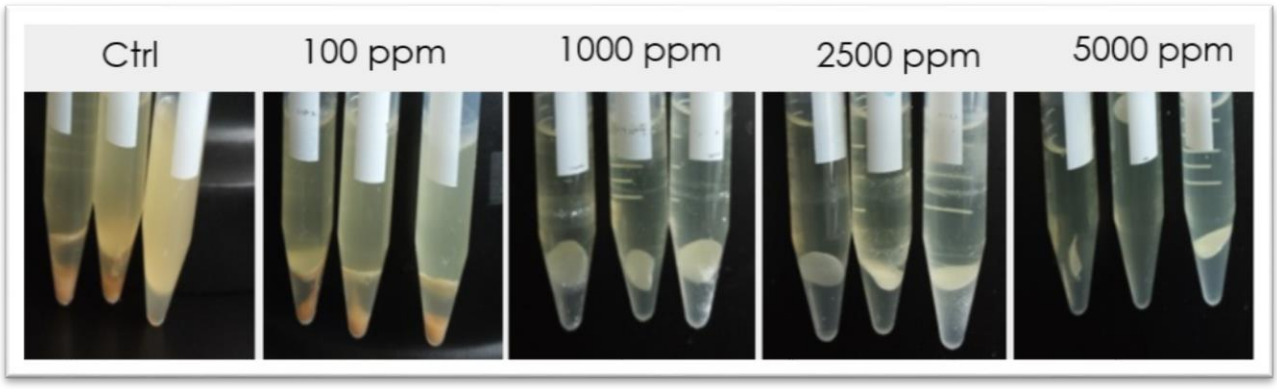

Fig 5: Sterilization efficacy of PAA on spore laden e-PCL scaffolds.

1.3.4 Effects of sterilization methods on physical and mechanical properties:

1.3.4a 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 6). 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 with controls.

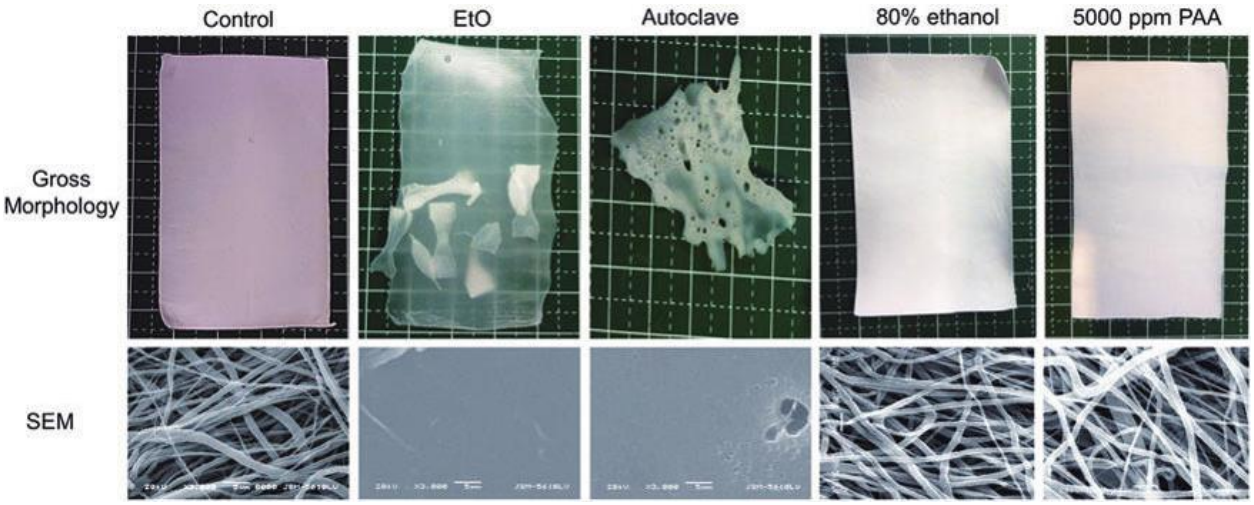

Fig 6: Effect of sterilization on gross morphology and SEM

The scanning electron micrographs of scaffolds treated with PAA diluted in DI water and 20% ethanol are shown in Figure 7. 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 fusing into bundles with evidence of fiber breakage at higher concentrations. PAA diluted in 20% ethanol showed a tendency toward thinning of fibers but preserved open porous architecture even at 5000 ppm. Statistical analyses confirmed significant effects of PAA

concentration on fiber diameter depending on 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 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 ($p > 0.8$).

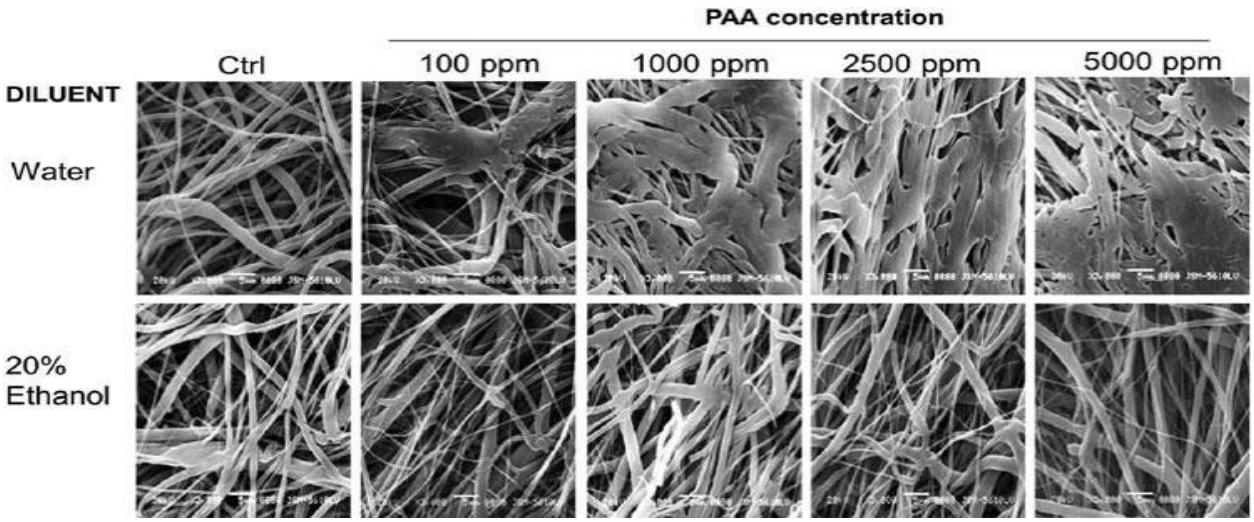

Fig 7: SEM of scaffolds treated with PAA diluted in DI water and 20% ethanol

1.3.4b Scaffold hydrophilicity: Contact angle measurements after different sterilization treatments were analyzed to indicate hydrophilicity or wettability of the scaffolds. Generally, surfaces are termed hydrophilic when the water contact angle is < 90^o and hydrophobic, if contact angle is more than 90^o. Figure 9 shows representative image of an actual drop placed on differently treated surfaces. Untreated control PCL scaffolds are highly hydrophobic (contact angle around 120^o); 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 a quantitative representation of the average of contact angles measured from six 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.

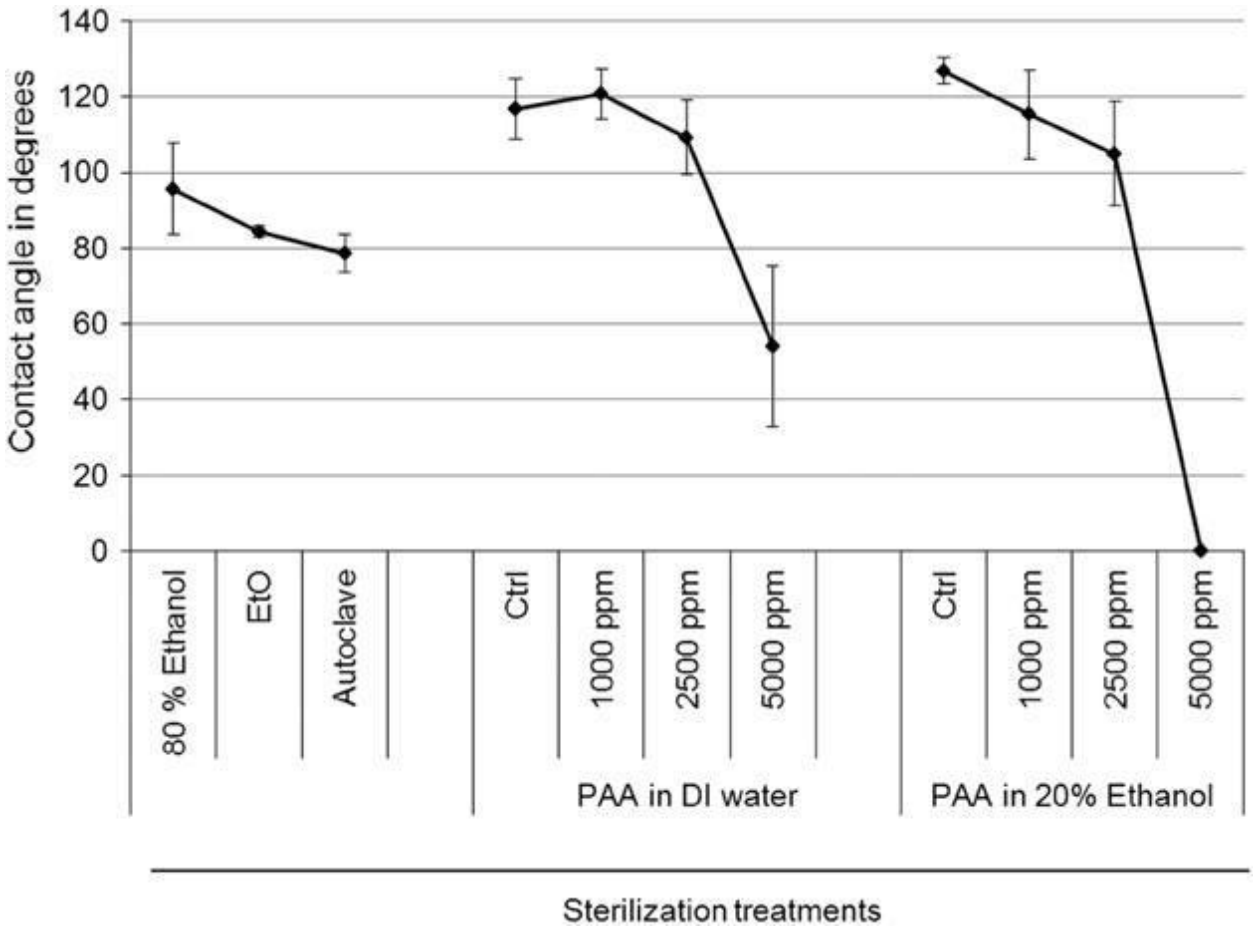

Fig 8: Quantification of contact angle measurements of PCL scaffolds

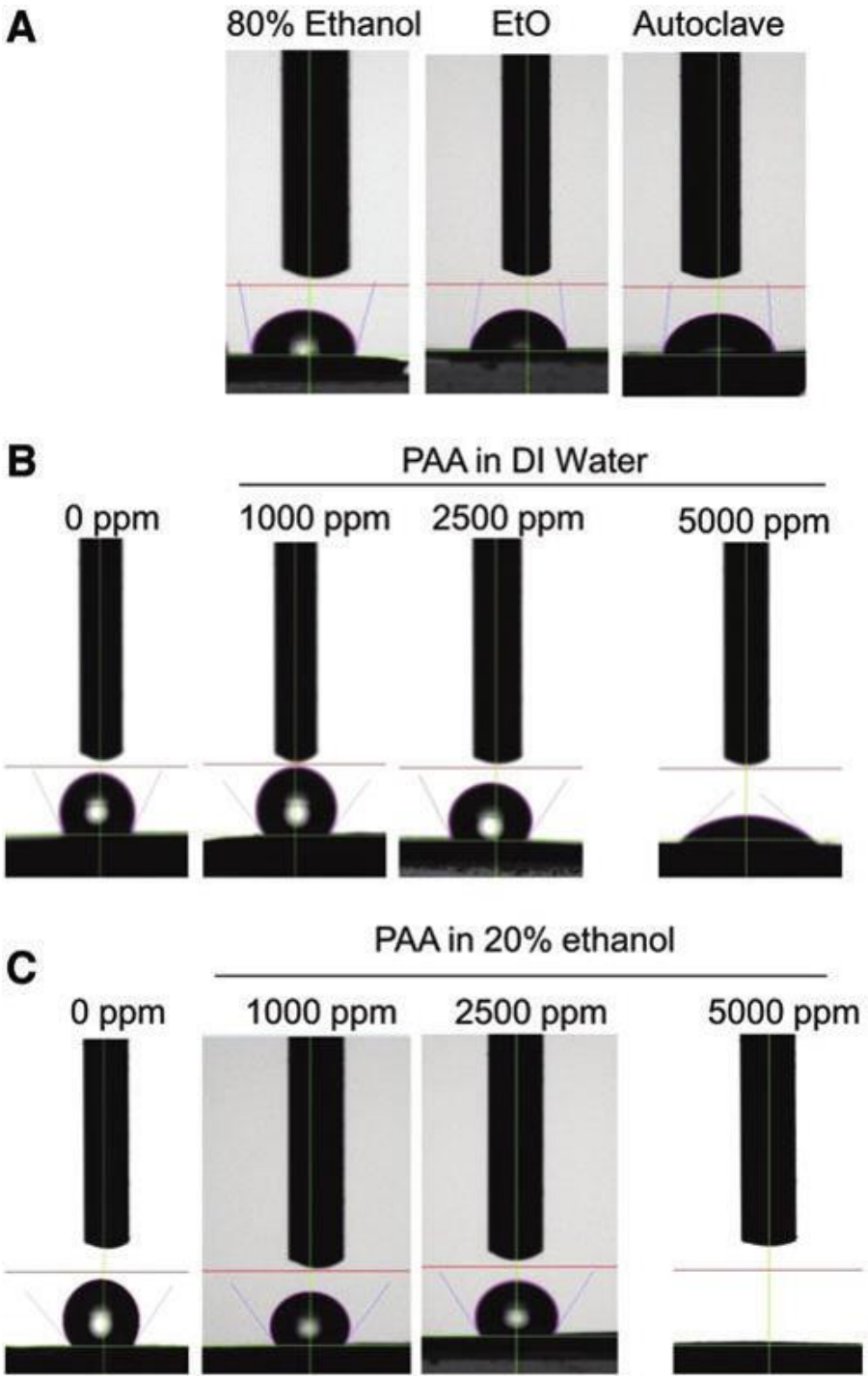

Fig 9: Sessile drop images, after 10 s equilibration, on e-PCL scaffolds subjected to standard sterilization methods (**A**) and PAA diluted in DI water (**B**) or 20% ethanol (**C**).While conventional treatment reduced contact angles appreciably, PAA did not have any significant effect for approximately 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.

1.3.4c 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 (Fig. 10, $p < 0.001$). 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 to 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).

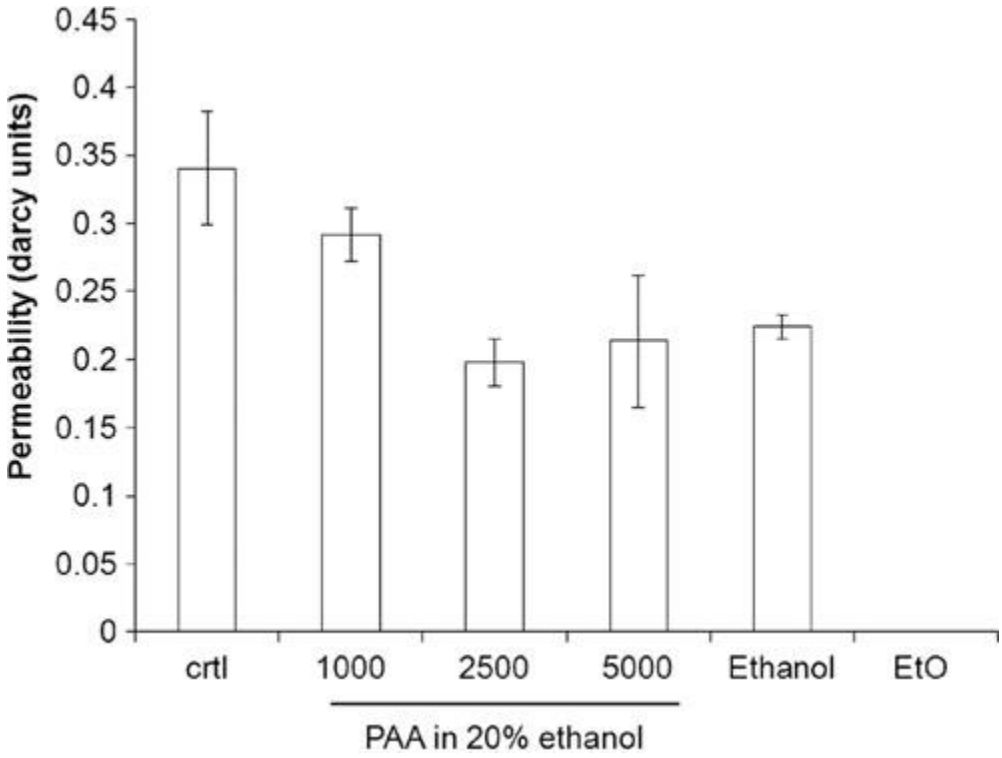

Fig 10: Scaffold permeability (measured in Darcy units) determined by flow rate of DI water through treated e-PCL scaffold.

1.3.4d 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, mechanical testing on these samples was not performed. The results of mechanical testing of scaffolds treated with 80% ethanol and different PAA concentrations are shown in Figure 11. 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 toward brittleness with increasing PAA concentrations for approximately 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).

1.3.5 PAA stability: PAA is at equilibrium with acetic acid and hydrogen peroxide and is particularly unstable at low concentrations. PAA at 100 ppm started degrading around 7 days as determined by a visual comparison with manufacturer-provided shade guides. 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 3 weeks.

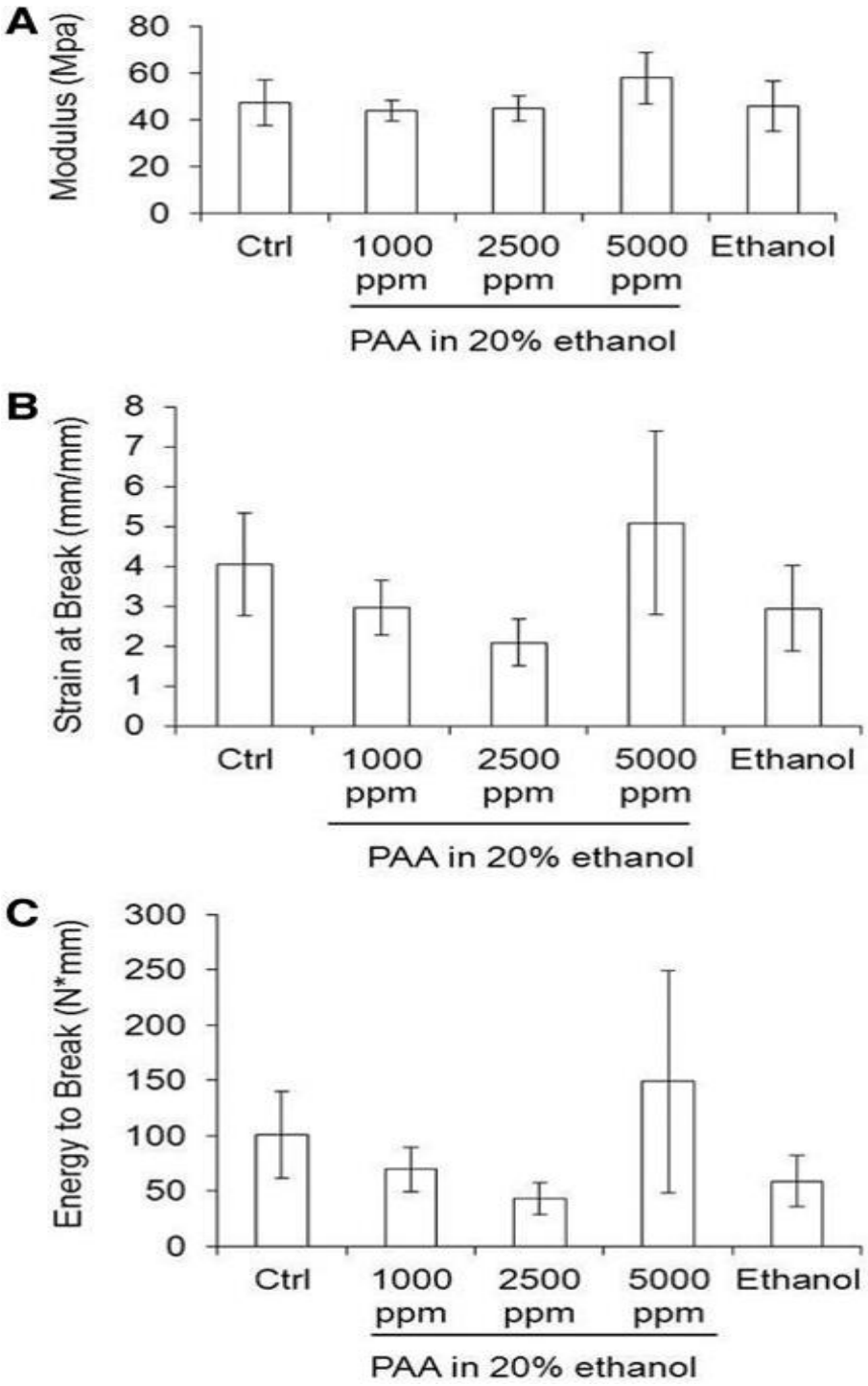

Fig 11: Mechanical properties of e-PCL scaffolds treated with different concentrations of PAA diluted in 20% ethanol. (A) Tensile modulus, (B) strain at break, and (C) energy to break. Control scaffolds refer to scaffolds incubated with 20% ethanol with no PAA. Scaffolds treated with 80% ethanol are also shown.

1.4 DISCUSSION

The aim of tissue engineering is to develop viable functional alternatives for failing/missing organs. However, the strategies pursued have evolved from purely cell- or biomolecule- based approaches to current paradigm of scaffold-based tissue engineering. This involves seeding and culturing specific cell types in engineered 3D matrices designed to simulate the native extracellular matrix[1]. Such matrices are expected to present appropriate physical, biological and biochemical cues to predictably influence cell behavior[18,19]. Synthetic polymers are popular in tissue engineering because they are biocompatible, biodegradable and are available in a wide range of properties. The growing list of polymers[2,20] and emerging scaffold fabrication technologies[21], provide matrices with range of internal architecture and mechanical properties.

Intended to be in direct contact with living tissues, these scaffolds must be terminally sterilized prior to implantation. One cannot assume product sterility even if fabricated in a 'clean room' because the machinery and starting materials are not sterile. In addition, normally benign bacteria can become pathogenic when present on the surface of devices. This makes scaffold sterilization an important issue that needs to be addressed 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. In addition, mechanical and surface properties, toxicity and biocompatibility of polymers needs to be evaluated before and after sterilization to ensure selection of an appropriate sterilization method that is benign to the polymer, device and the patient. 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^{o}C for 15 minutes is not practical for sterilizing polymers with low melting points. In addition, most biocompatible polymers are hydrolytically unstable and exposure to moisture can accelerate degradation, reduce shelf life and negatively affect physical properties[22]. EtO Is a reactive gas that can penetrate into polymeric networks, react with its chemical groups, cause degradation of polymer and alter scaffold dimensions[23]. In addition, EtO is carcinogenic and needs to be extensively degassed over many hours prior to packaging[15]. High energy irradiation is an efficient sterilization method that may preserve the morphology of a 3-D scaffold, but it dramatically decreases the polymer molecular weight and hence, accelerating degradation[10].

Limitations of conventional modes of sterilization in tissue engineering have led researchers to explore alternatives especially in the past few years. Shearer et al.[24] found that peracetic acid 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[25]. 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[26] evaluated the effects of sterilization on lyophilized sericin-polyvinyl alcohol scaffolds and concluded that gamma irradiation as the most appropriate method even though it degraded by almost 70% in 24 hours. Even though these studies are very important, the authors do not specify the source or the identity of the contaminating bacteria in many cases thus making comparisons

difficult. Mostly, the groups used unsterilized material as control which can vary widely in their bacterial load or bio-burden. This study sought to address this issue by using B. *atrophaeus* spores as biological indicators.

The use of chemical agents to reduce bacterial load in polymeric scaffolds is attractive because it allows processing at low temperatures and rapid processing. Since sterilization provides a higher standard of care as well as highest margin of safety for patients, we inoculated 10^6 spores of B. *atrophaeus* onto electrospun PCL scaffolds to serve as biological indicators. Spores, being the more resistant form of life and present in large numbers, a negative spore test would mean complete elimination of bio-burden[27] and a sterile scaffold.

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 and disrupts cell wall permeability and is effective against all forms of microbes (including spores) even in the presence of organic matter[28]. PAA is effective at low concentrations, low temperatures and reduced contact times. PAA is also economical, degrades into non-toxic (water, oxygen and carbon-di-oxide) end products and can be safely disposed down the drain without affecting the environment[27].

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

A significant deviation from FDA approved STERIS protocol to account for differences in biomaterials employed. First, since polymeric scaffolds are sensitive to heat (PCL degraded with EtO exposure at 50^{o}C) and the priority was to effect sterilization at room temperature. Second, in contrast to traditional solid surfaces (tubes and instruments), engineered scaffolds are three dimensional, nanofibrous porous structures. The enormous surface to volume ratio offered by electrospun scaffolds is a huge benefit for tissue engineering but also presents a problem for sterilization due to the potential for lodging and survival of spores/ bacteria in the depth of the scaffold.

The baseline conditions for sterilization in terms of contact time and temperature was established. PAA when added to spore suspension was effective in 5 minutes at room temperature; however, taking into account the 3D porous nature of the electrospun scaffold, the contact time was increased to 15 minutes for all scaffold based experiments. Working with various dilutions of PAA, an observation was made; wettability of the scaffold affected the ability of PAA to kill spores. Since the 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 without affecting the sporicidal activity of PAA at previously established concentrations. This is consistent with the fact that PAA need to be in physically contact to effect sterilization.

An interesting effect of PAA at high concentrations on surface properties of electrospun scaffolds was detected. Control PCL scaffolds were significantly hydrophobic as reflected by the contact angle measurement of greater than 120 degrees. Contact angles tend to decrease with increasing concentrations of PAA even though the differences were not statistically significant. However, at 5000 ppm, the scaffold surface became so hydrophilic that that the contact angle

decreased to zero with the scaffold literally soaking up the drop. In order to determine if this observed effect is due to the diluent (20% ethanol) or PAA, the experiment was repeated with no diluent. The contact angle did not drop to zero but it was significantly less than controls. In both cases, the scaffolds became hydrophilic. This is not unexpected because polyester polymers are known to undergo acid- or alkali mediated hydrolysis beyond a threshold pH. It is an interesting observation because conscious efforts are being made to improve the hydrophilicity of scaffolds as it directly affects biocompatibility and favorable host response[30,31]. This finding makes PAA attractive because of its ability to sterilize as well as to improve the surface properties of electrospun scaffolds.

Since acid-mediated hydrolysis can potentially affect morphological characteristics and mechanical properties of electrospun scaffolds, the study sought to evaluate these effects by SEM and tensile testing. The results portray no alteration in the sub-micron level architecture when scaffolds were treated with 5000ppm PAA diluted in 20% ethanol. However, the fibers tend to fuse and scaffolds demonstrates decreased porosity when DI water was used as a diluent.

The study presented has the following limitations: working with one representative low-melting polymer (PCL) fabricated by electrospinning. Even though we expect the results to be valid in other polymer systems and scaffold fabrication technologies, the results cannot be directly extrapolated and needs to be treated with caution. Conditions for sterilization and effects on scaffolds will vary and need to be optimized for specific systems. Cell response to sterilized scaffolds is an important issue and the second chapter will address the results of *in vitro* experiments.

1.4 CONCLUSIONS:

The primary goal was to systematically evaluate the feasibility of using peracetic acid (PAA) as an effective technique to sterilize electrospun polymeric scaffolds. PCL was deliberately chosen because it represents low melting polymers (55-60^{o}C) and is popular in tissue engineering. Even though few prior studies were done to evaluate the effects of sterilization on engineered scaffolds, this is the first to employ spores as a challenge organism. In addition, electrospinning allowed generation of porous, nanofibrous scaffolds thus providing the opportunity to observe the effects of sterilization on scaffold architecture at nanoscale. The quest was to identify optimal conditions that would 1.sterilize the scaffold at normal temperature and pressure, 2. Preserve the nanofibrous morphology and 3. Maintain or improve mechanical/ surface properties. PAA at 1000 ppm for 15 minutes at room temperature renders the scaffold sterile without having any adverse effects on the morphological or mechanical properties of the scaffold. Incubation in 5000 ppm PAA for 15 minutes renders the scaffold hydrophilic as well. The role of diluent is important and is shown to significantly affect the sterilization efficacy and alter scaffold architecture. PAA diluted in a solution of 20% ethanol seems to be able to retain the sporicidal properties without affecting scaffold attributes.

Comparison of Osteoblast Survival on Electrospun Polycaprolactone and Alvetex™ Scaffolds Following Peracetic Acid Sterilization

Chapter 2

2.1 INTRODUCTION:

Restoration of structure and function of failed tissues is essential of maintenance of quality of life. None of the current therapies (auto/allo grafts, mechanical devices, and artificial prostheses) result in satisfactory long-term outcomes[32]. Tissue engineering is a promising strategy that attempts to replace missing or diseased tissues by implanting natural, synthetic, or semisynthetic tissue and organ mimics that are intended to develop functionality over time[33]

The three fundamental components of tissue engineering are scaffolds, cells and signaling molecules. Scaffolds are structures with defined three-dimensional form that simulates the natural extracellular matrix[34]. Scaffolds provide microenvironment that mimics the biochemical and mechanical aspects of natural extracellular matrix. Foremost among the list of requirements of implanted tissue engineered scaffold is its cytocompatibility. Beyond this basic requirement, the scaffolds need to be conducive to cell attachment, migration, proliferation, differentiation and maintenance of cell phenotype[35,36,37].

Synthetic resorbable polyesters are popular choice in scaffold fabrication because of the ability to tailor mechanical properties and degradation. Additionally, the micro/nano architecture can be controlled by employing different fabrication methods[38],[39]. Electrospinning is a versatile scaffold fabrication technology that consistently reproduces the fibrous morphology of the native ECM. In addition, the process is scalable; allows control over fiber diameter[40,41], porosity[42,43] and alignment[44],[45]. Sterilization of tissue engineering scaffolds is an important regulatory issue and is mandatory prior to clinical translation. A majority of published literature in tissue engineering involves use of ethanol or UV to disinfect scaffolds; methods that are clearly inadequate for human use. The previous study has revealed that conventional methods of sterilization cause loss of

structural integrity in electrospun scaffolds made from low-temperature melting polymers while chemical sterilization using peracetic acid (PAA) preserved its fibrous architecture and mechanical properties[46]

Unlike physical methods (radiation and heat) that are terminal, chemical sterilization (liquid or gas) need to be followed by a decontamination regimen to eliminate residuals. Adsorption of cytotoxic and carcinogenic ethylene oxide (EtO) residuals onto polymeric surfaces is well acknowledged and extensive aeration is required prior to clinical use[27]. Tissue engineering scaffolds must support cell attachment and growth at the implanted site to be effective. It is therefore important to ensure all toxic residuals are eliminated post-sterilization. The previous study demonstrated that PAA can effectively sterilize PCL scaffolds and this study focuses on evaluating the biological effects of PAA sterilization using mouse calvarial osteoblasts (MC3T3). This chapter compares the cellular effects on two different substrates: electrospun PCL scaffolds and commercially available polystyrene scaffold (Alvetex™).

2.2 METHODS:

2.2.1 Electrospinning PCL Scaffolds: PCL (Sigma, MW 80,000) was dissolved in a binary solvent system of formic acid: acetic acid (1:3 ratio) at a concentration of 100 mg/ml[47]. Electrospinning apparatus (EC-DIG, IME Technologies, The Netherlands) was used at optimized process conditions (rate: 2 ml/h, air-gap distance: 12.5 cm, applied needle voltage: +25 kV) to generate continuous, non-woven fibers that were collected onto 18mm glass cover slips attached to cylindrical drum mandrel (100 mm diameter) rotating at 100 rpm. After electrospinning, cover slips were removed from the mandrel, dried in a fume hood overnight and stored in an airtight desiccator until use.

2.2.2 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 platinum 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). Cells seeded scaffolds were fixed in 2.5% glutaraldehyde, sequentially dehydrated in ethanol followed by 3 minute incubation in hexamethyldisilazane (HMDS, Sigma) prior to imaging[48].

2.2.3 Scaffold Sterilization: The scaffolds on coverslips were sterilized using previously identified conditions for PAA sterilization. Stock PAA (390,000 ppm) was diluted in 20% ethanol to 1000 ppm and the scaffolds were incubated for 15 min at room temperature (PAA-PCL) while control scaffolds (Ctrl-PCL) were disinfected with 70% ethanol for 30 min. All scaffolds were washed thrice with PBS (10 min each) and incubated in cell culture media overnight prior to cell seeding.

2.2.4 Cytotoxicity Assay: Live/Dead assay (Life Technologies) was used to evaluate cytocompatibility of PAA-PCL before and after different neutralization methods (see sections 2.5-2.8). A total of 20,000 MC3T3 cells were seeded at the center of scaffold and retained within a 10mm diameter glass cloning ring (Corning). Cell constructs were cultured in DMEM with 10% FBS (Atlanta Biologicals, GA) and 1% antibiotic/antimycotic (Invitrogen) at 37°C and 5% CO_2. All Live/Dead experiments were done in triplicate; assays was performed at 24 hours and imaged using Nikon fluorescence microscope (10X). Live MC3T3 cells with esterase activity appear green while dead cells with exposed DNA emit red fluorescence. All images are overlay of green and red channels indicating live and dead cells respectively. Live/Dead assay was used as a screening tool to assess early cytotoxicity.

2.2.5 Neutralization Using STS and Catalase: PAA is an equilibrium mixture of peracetic acid (39%), hydrogen peroxide (6%) in acetic acid. Since PAA is a strongly oxidizing, its neutralization was investigated using 20mg/ml of sodium thiosulfate (STS) for 2 hours[49], a known reducing agent. Additionally scaffolds were incubated in catalase (0.1mg/ml) for 8 hours[50], an enzyme known to effectively quench hydrogen peroxide.

2.2.6. Aeration of PAA-PCL: A modified protocol traditionally used in decontamination following EtO sterilization. PCL being a low melting polymer aeration was carried out at room temperature. PAA-PCL scaffolds were placed in an aeration chamber for 18 hours, treated with STS (20 mg/ml) and catalase (0.1mg/ml) prior to cell seeding.

2.2.7. PAA Quenching using Ethanol: Since ethanol treatment of scaffolds improves wetting characteristics, it was hypothesized that washing PAA-PCL will effectively desorb residues. Accordingly, PAA-treated scaffolds were quenched in 70% ethanol for 30 minutes followed by washing in PBS, prior to cell seeding.

2.2.8. Cell Proliferation Assay: Cell proliferation was evaluated using MTS (Promega) assay on two different scaffold types: electrospun PCL and commercially available Alvetex polystyrene scaffolds (Reinnervate Ltd. Durham, UK). Four groups were accordingly identified: electrospun PCL scaffolds treated with PAA (PAA-PCL), PAA-treated electrospun scaffolds quenched with ethanol (PAA-PCL+EtOH), Alvetex scaffolds treated with PAA (PAA-Alv) and PAA-treated Alvetex quenched with ethanol (PAA-Alv+EtOH). Control scaffolds were disinfected with 70% ethanol and designated Ctrl-PCL and Ctrl-Alv. In addition, tissue culture polystyrene (TCPS) was included for comparison. All scaffolds were washed thrice in PBS for 10 minutes and incubated in cell culture media overnight prior to cell seeding. A total of 10,000 MC3T3 cells were seeded onto scaffolds and a modified MTS assay (Promega, Madison, WI) performed on days 1 and 7. Briefly, cell-seeded scaffolds were washed with PBS at designated time points and incubated with MTS reagents for 2 hours. The absorbance of the supernatant was read at 490 nm using BioTek Synergy 2 microplate reader. Experiments were performed in triplicates and repeated once to confirm the results. The optical density (OD) was analyzed using a mixed-model, repeated-measures ANOVA. Since absolute values of OD were skewed, the data were analyzed on log scale and then transformed back to original units using SAS software (SAS Institute, Inc., Cary NC). The data is graphically represented as means with 95% confidence intervals (CI). Statistical significance was set a priori at 0.05.

2.3 RESULTS:

2.3.1 Scaffold Characterization: The scaffolds generated from binary solvent system-FAA (formic and acetic acid) predictably yielded fibrous scaffolds with a mean fiber diameter of 210 nm (Figure 1).

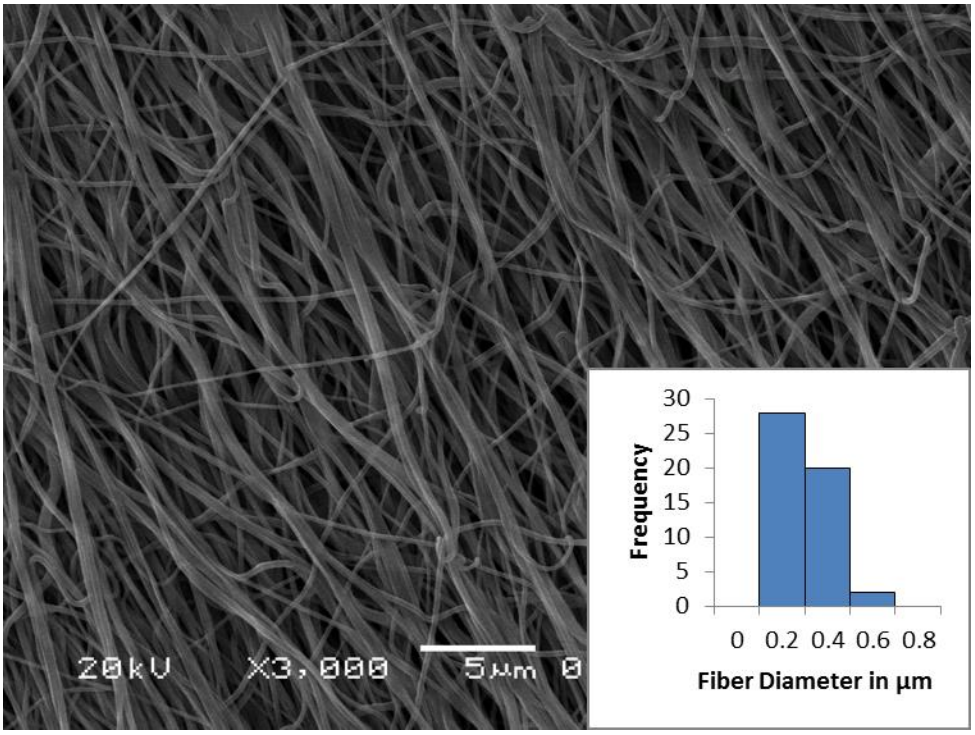

Fig 1: SEM micrograph of electrospun PCL generated from binary solvent system-FAA (formic and acetic acid)

2.3.2 Cell Survival on PAA-PCL: The results of Live/Dead assay on PAA-treated scaffolds and control scaffolds are presented in Figure 2. While Ctrl-PCL supported robust cell attachment and survival, PAA-PCL was completely cytotoxic: significantly less number of cells attached and any entrapped cell was dead within 24 hours.

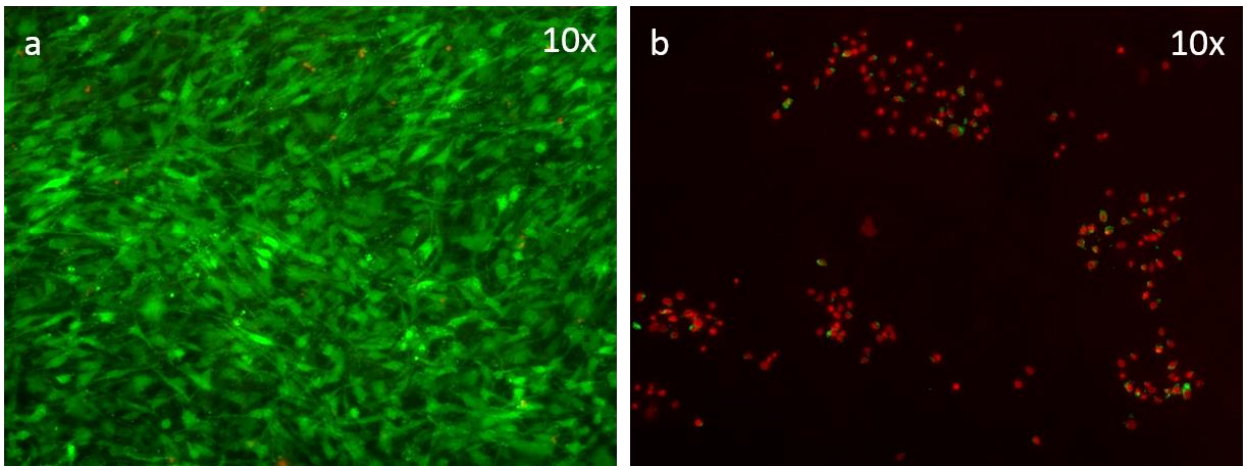

Fig 2: Effect of PAA on cell viability. Live/Dead staining of MC3T3 on Ctrl-PCL (a) and PAA-treated scaffolds (b)

2.3.3 STS and Catalase Neutralization on PAA-PCL: Incubation of PAA-scaffolds with 20mg/ml of STS did not improve the cytocompatibility profile and neither was STS cytotoxic (Figure 3). These results show that thiosulfate was not adequate or incapable of neutralizing adsorbed PAA residuals. There was an increase in the number of attached cells as well as a higher proportion of live cells when the scaffolds were incubated with 0.1mg/ml catalase (Figure 4). However, higher concentrations of catalase did not improve survival. These results suggest that adsorbed hydrogen peroxide contributed partially to cytotoxicity in PAA-treated scaffolds and that catalase was able to quench adsorbed hydrogen peroxide residuals.

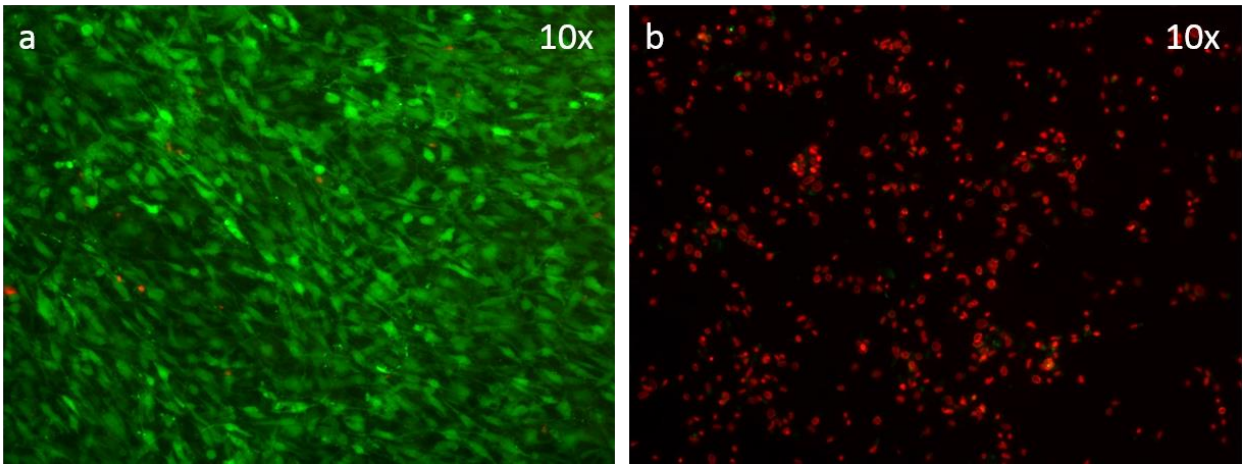

Fig 3: Effect of PAA neutralization by STS on cell viability. MC3T3 on Ctrl-PCL incubated in 20mg/ml STS is shown on the left (a) while PAA-treated scaffold is shown in panel (b).

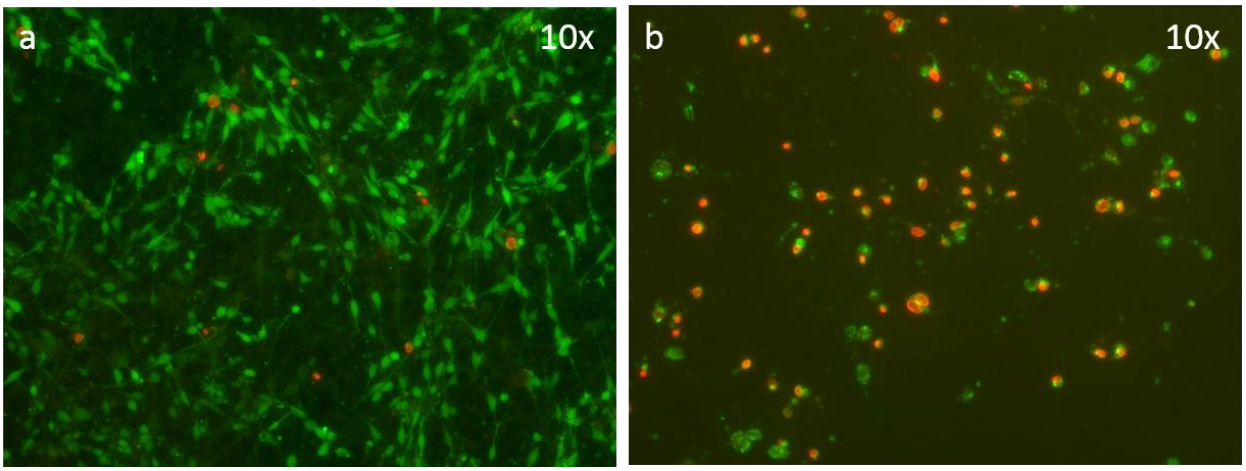

Fig 4: Effect of catalase on cell viability following PAA exposure on electrospun scaffold. Live/Dead images from Ctrl-PCL (a) and PAA-PCL (b) treated with 0.1mg/ml catalase

2.3.4 PAA Aeration: PAA-PCL aerated for 18 hours, showed a remarkable decrease in the number of dead cells with a concomitant increase in living cells even though the cell density is significantly less than the controls (Figure 5) suggesting this strategy was moderately effective in improving scaffold cytocompatibility.

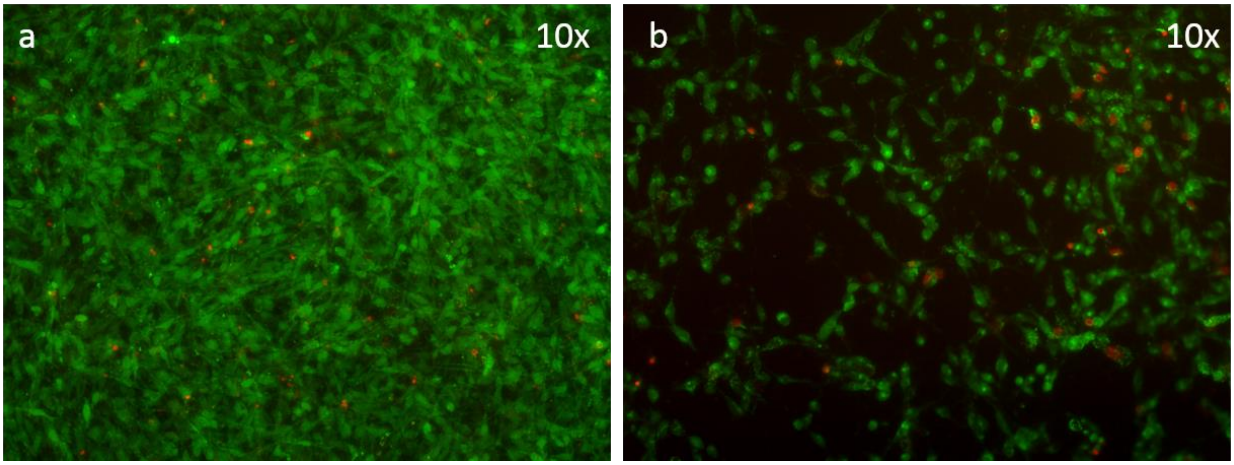

Fig 5: Effect of PAA decontamination by aeration, STS and catalase. Live/Dead image of MC3T3 on Ctrl-PCL (a) and PAA-PCL (b).

2.3.5 PAA Quenching with Ethanol: The effect of quenching PAA-PCL in 70% ethanol for 30 minutes (PAA-PCL+EtOH) is illustrated in Figure 7. As can be seen, ethanol treatment fully restored the scaffold cytocompatibility to levels comparable to controls. The cell density, flattened cell morphology and the proportion of live cells were similar to that of Ctrl-PCL. Comparable cell spreading behavior on Ctrl-PCL and PAA-PCL+EtOH was evident in scanning electron microscopy (Figure 6) confirming reversal of cytotoxicity with treatment.

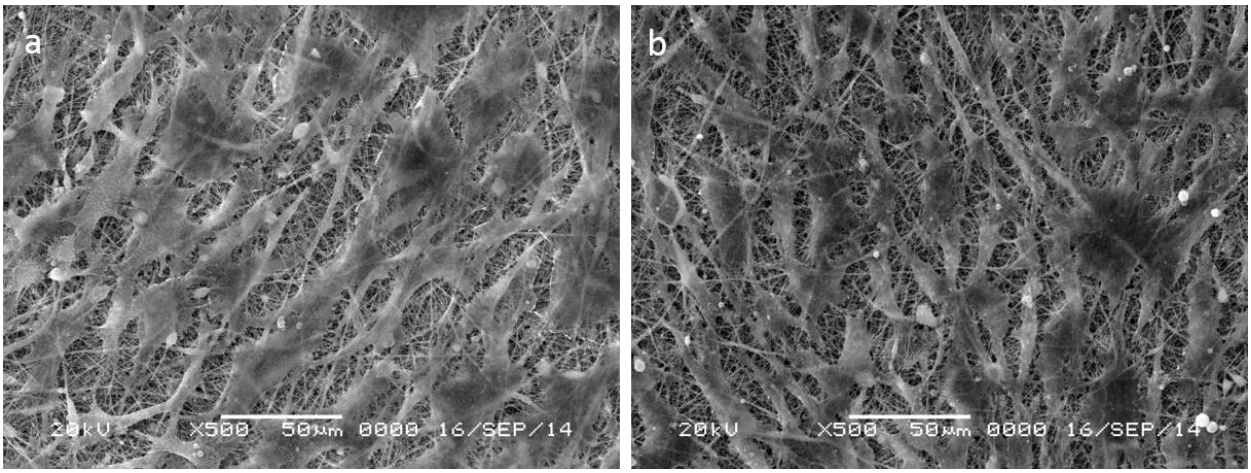

Fig 6: SEM images of MC3T3 after 24 hours of culture on Ctrl-PCL (a) and PAA-PCL+EtOH (b)

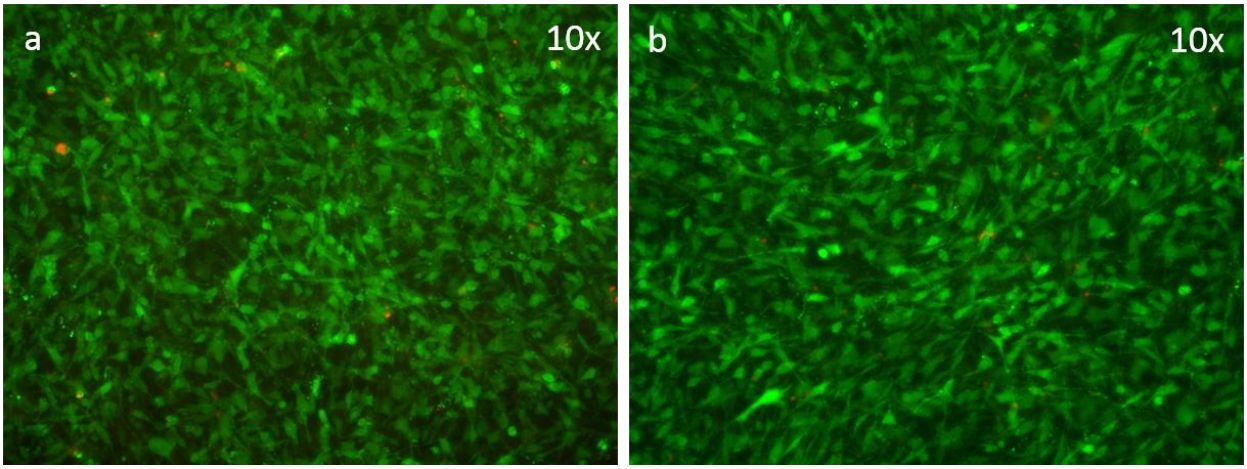

Fig 7: Effect of PAA quenching with 70% EtOH; Live/Dead image of MC3T3 on Ctrl-PCL (a) and PAA-PCL quenched with EtOH (b).

2.3.6. Cell Proliferation (MTS) Assay: Standard curve for MTS assay was established using MC3T3 cells (R2=0.9981) to validate the assay protocol. As seen in Figure 8 there is a significant increase in cell numbers from day 1 to day 7 in all groups with the exception of PAA-PCL group. This is due to profound cytotoxicity associated with PAA treatment of electrospun PCL scaffolds (confirmed by previous Live/Dead results). More importantly, there is no statistically significant difference in the day 1 cell viability between TCPS, Ctrl-PCL and PAA-PCL+EtOH groups ($p=0.9079$). This indicates complete restoration of cytocompatibility after exposure of electrospun PCL to PAA. Despite seeding the same number of cells, there were significant differences in OD between Ctrl-PCL and Ctrl-Alv on day 1. Even though inherent differences between these substrates can account for these observations, this also presents a difficulty in comparing proliferation data. Therefore a logical approach of comparing was adapted the OD ratios at these time points (day7: day1). This strategy eliminated the tendency of baseline differences to skew the data and provided a means to objectively evaluate scaffold performance over time (Figure 9). The cells nearly doubled (ratio 1.96) in 7 days on TCPS and this increase is statistically significant

compared to Ctrl-PCL (ratio nearly 1.6). While PAA-PCL+EtOH group show significantly improved cell number over time (day 1 and day7), the rate of cell proliferation is still significantly less than TCPS group (p=0.013). In complete contrast to electrospun scaffolds, Alvetex scaffolds showed significantly enhanced cell proliferation following PAA exposure. Interestingly, if PAA-treated Alvetex scaffolds were washed with ethanol (PAA-Alv+EtOH), the proliferation rates return back to baseline (Ctrl-Alv group), suggesting that ethanol is very effective in desorbing adsorbed residuals from PAA sterilization.

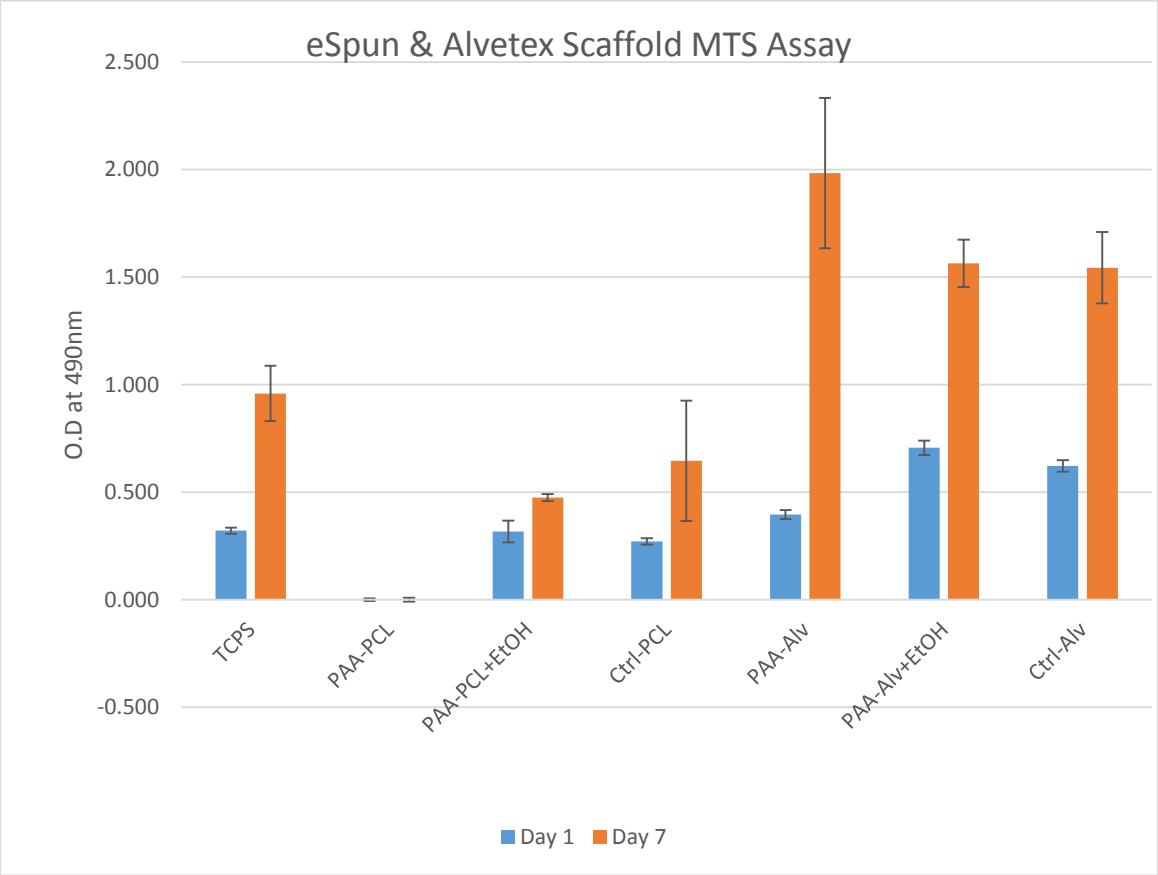

Fig 8: Cell Viability at days 1 and 7 as determined by optical density (OD) of MTS assay.

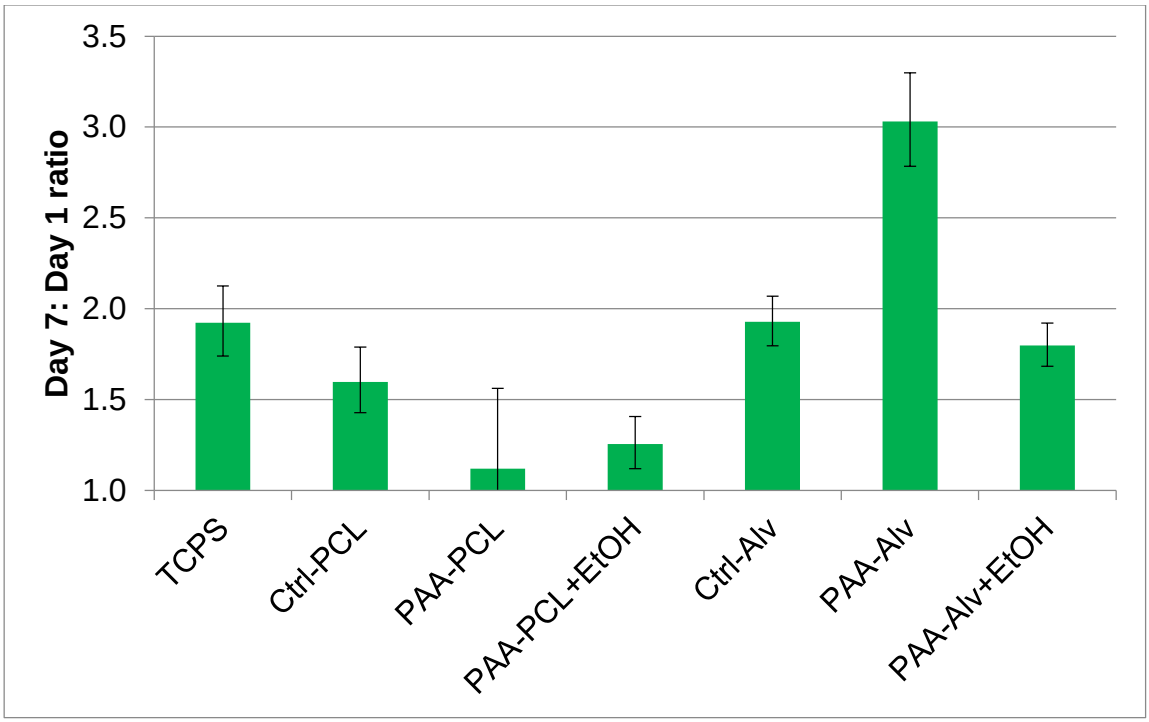

Fig 9: Ratios of OD from day 7 to day 1 to illustrate ability of the scaffold to support cell proliferation

2.4 DISCUSSION

The ever-increasing use of biomaterials as components of surgically implanted devices makes it imperative that they are evaluated for safety or toxicity prior to clinical application. Tissue engineering scaffolds represent a unique class of biomaterials that are surgically placed in the defect site and are intended to integrate, remodel and eventually replaced by host tissue. The most fundamental requirement of such biomaterial constructs is their biocompatibility (i.e.) their use should not impose any unnecessary adverse or toxic response in a patient/ biologic environment.

The most common cause of incompatibility of a biomaterial is the presence of biologically active substances that are absorbed within, condensed or adsorbed upon the surface of the polymer. Upon contact with biological fluids or media, substances absorbed into the matrix migrate to the surface where they are solubilized and released into the immediate environment to induce toxic effects. Since toxic leachables can arise as sterilant residues (liquid or gas sterilants) or degradation products, it is important to identify the leachable as well as its source to successfully eliminate them from the biomaterial.[57]

In the present study, PCL (MW 80000) was used as the starting material for electrospinning scaffolds from a binary solvent system of formic acid: acetic acid. Test scaffolds were subjected to PAA sterilization while control scaffolds were disinfected with 70% ethanol. Following sterilization/ disinfection treatment, MC3T3 cells were cultured on both scaffold types under identical conditions. The data shows robust cell survival and growth in control scaffolds while PAA-PCL induced massive cell death within 24 hours and hence was not cytocompatible. The observations that (1) PAA-PCL induced an acute cell death, (2) PAA and its components are known corrosives[29], and have been shown to absorb onto polymers[51] clearly reflect the sterilant residues as the cause of observed toxicity. Since PAA was shown to achieve effective sterilization

of low-temperature melting, hydrolysable polyester PCL (m.p. 59^{o}C) at room temperature, the aim was to identify suitable post-sterilization process/modification to eliminate these toxic leachables.

Absorption and adsorption are phenomena that result in uptake of substance from the medium onto solid biomaterial and are observed with many polymers. While adsorption is a surface phenomenon that is easily reversed, absorption is a bulk phenomenon and such molecules are not easily separated from the absorbent. Radl et al. tested three different polymers and reported highest amounts of absorption of hydrogen peroxide by polyvinyl chloride during sterilization. Further, desorption kinetics from polymers showed strong dependence on material composition and temperature[51]

Results presented are in agreement with the results of Franklin et al.[52], who reported significant cytotoxicity associated with low-temperature hydrogen peroxide gas plasma treatment of PCL scaffolds. The authors suggested that leaching of residual hydrogen peroxide from within or upon the porous scaffolds could explain observed cytotoxic effects and concluded that oxidizing plasma treatment is not compatible with 3 dimensional, porous PCL scaffolds. While we observed similar cytotoxic effects, suitable modification of the post-sterilization processing conditions led to complete recovery of cytocompatibility.

In order to evaluate if the PAA induced cytotoxicity was substrate dependent, a comparison experiment was set up between electrospun PCL scaffolds and Alvetex, a commercially available polystyrene scaffold. The latter scaffolds, generated by high internal phase emulsion, have porosity >90% with regular (~40um) interconnected pores[53,54]. In complete contrast to e-PCL scaffolds, PAA treatment of Alvetex resulted in significantly enhanced biologic performance; interestingly, these effects were promptly reversed when Alvetex was quenched in ethanol. These results could be attributed to reversible changes in the orientation of polymer chains, induced by PAA to favor

cell attachment. The fact that PAA-induced changes are dependent on substrate chemical composition is also supported by a recently published study[55] that reported no cytotoxicity in PAA-treated, decellularized, cadaveric tendons.

Not surprisingly, we found that available surface area plays a significant role in the toxicity of electrospun PCL scaffolds. PCL was electrospun from two different solvents, hexafluro-isopropanol (HFP) and a binary solvent system of formic acid:acetic acid (FAA) to yield scaffolds with distinct fiber diameters. While HFP generated fibers with mean diameter of 820 nm (range: 135 - 2030nm), FAA generated fibers with average diameter of 219 nm (range: 113 - 468nm). Specific surface areas (total surface area/ total volume) for each scaffold type were calculated following previous published protocol[56]. Scaffolds from HFP had specific surface area four times lower than their counterparts from FAA. In order to investigate the increased specific surface area on absorption of sterilant residues, both scaffold types were treated with PAA as described in the methods section and evaluated for 24-hour cytotoxicity. While both of these scaffolds did not support cell survival as strongly as controls, significantly higher cytotoxicity was found in PCL electrospun from FAA compared to those generated from HFP (Figure 10)

Even though cytotoxicity on MC3T3 was used as a surrogate marker to evaluate the presence of PAA residuals, its use is justified because it represents a valid marker of cytocompatibility and accurate quantification of absorbed residuals is challenging.

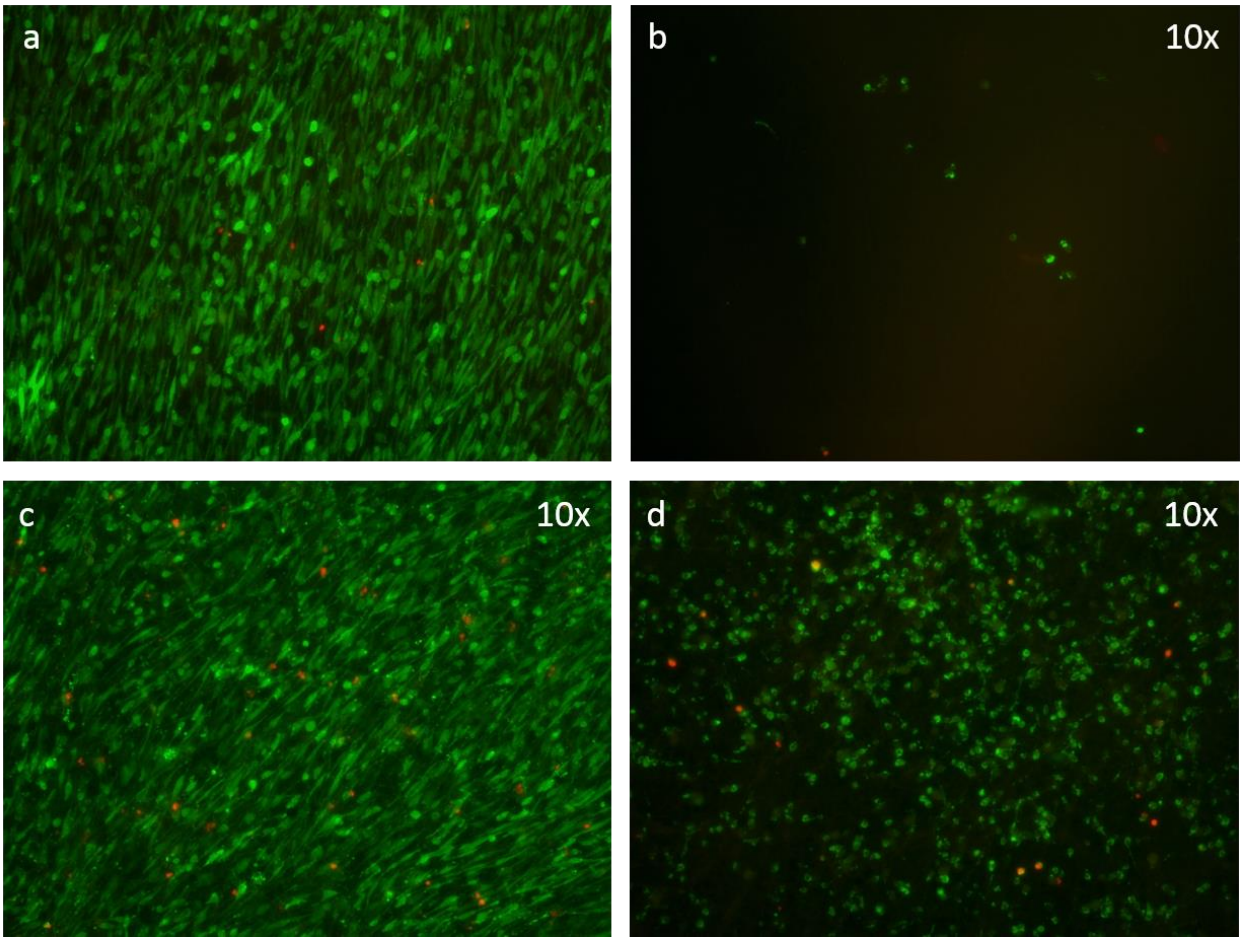

Fig 10: Live Dead Images of MC3T3 depicting higher cytotoxicity in PCL electrospun from FAA vs HFP; Ctrl-PCL [FAA] (a), Ctrl-PCL [HFP] (c), PAA sterilized PCL generated from FAA and HFP (b) and (d) respectively.

2.5 CONCLUSION

Liquid or gas sterilization technologies, while effective, are associated with of toxic residuals at the end of sterilization process. The issue is even more pertinent with polymeric biomaterials because of their ability to absorb chemical sterilants during processing. Release of sterilant residuals from the biomaterial is the common cause of acute toxicity in a biological environment. The observed acute cytotoxicity in PAA sterilized electrospun PCL scaffolds, suggesting incomplete elimination of absorbed residuals. Since PAA was previously shown to achieve effective sterilization of electrospun PCL at room temperature without affecting scaffold properties, strategies were investigated to eliminate residuals after PAA sterilization. While conventional neutralization methods (STS, catalase, aeration) were ineffective, quenching PA-PCL in 70% ethanol for 30 minutes resulted in complete elimination of toxic residuals and restored scaffold cytocompatibility. In complete contrast to electrospun PCL, Alvetex scaffolds showed significantly enhanced cell attachment and proliferation after PAA treatment. Interestingly, this enhanced biological response was abolished following ethanol quenching indicating this effect was specific to PAA treatment. These data illustrate that biological effects of PAA sterilization depends on substrate chemistry as well as scaffold architecture. PAA thus presents an interesting opportunity to sterilize low-temperature melting polymeric scaffolds but constructs should be evaluated for toxic residuals prior to extensive testing.

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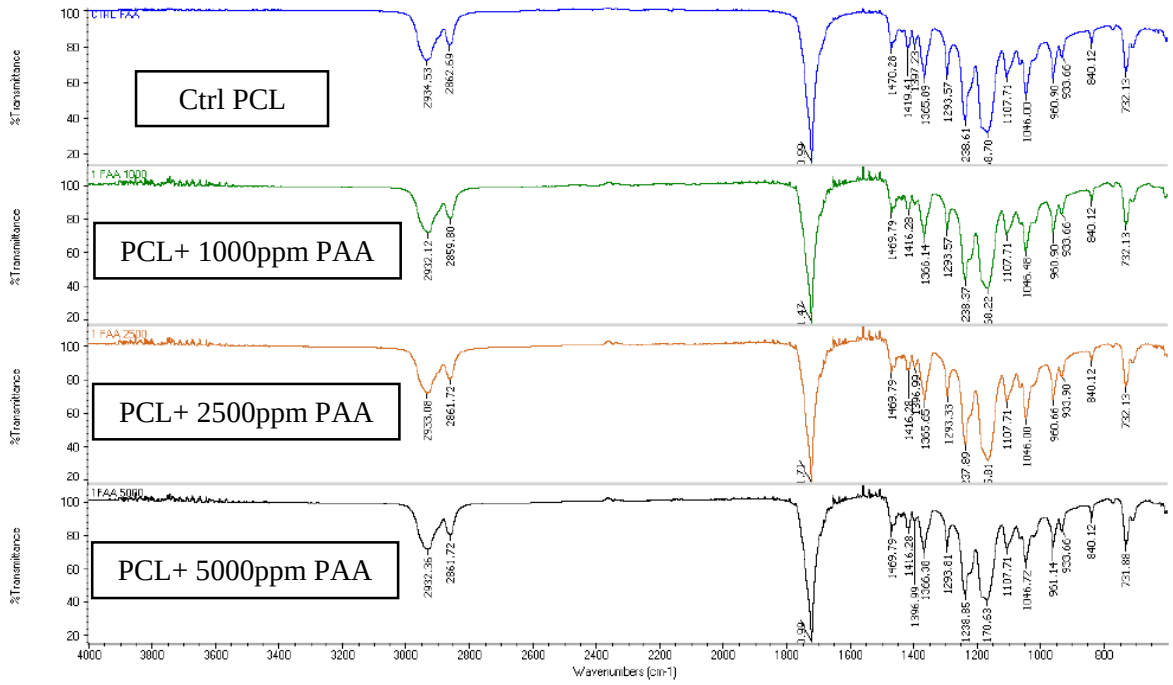
SUPPLEMENTARY MATERIAL

1) Fourier transform infrared spectroscopy data:

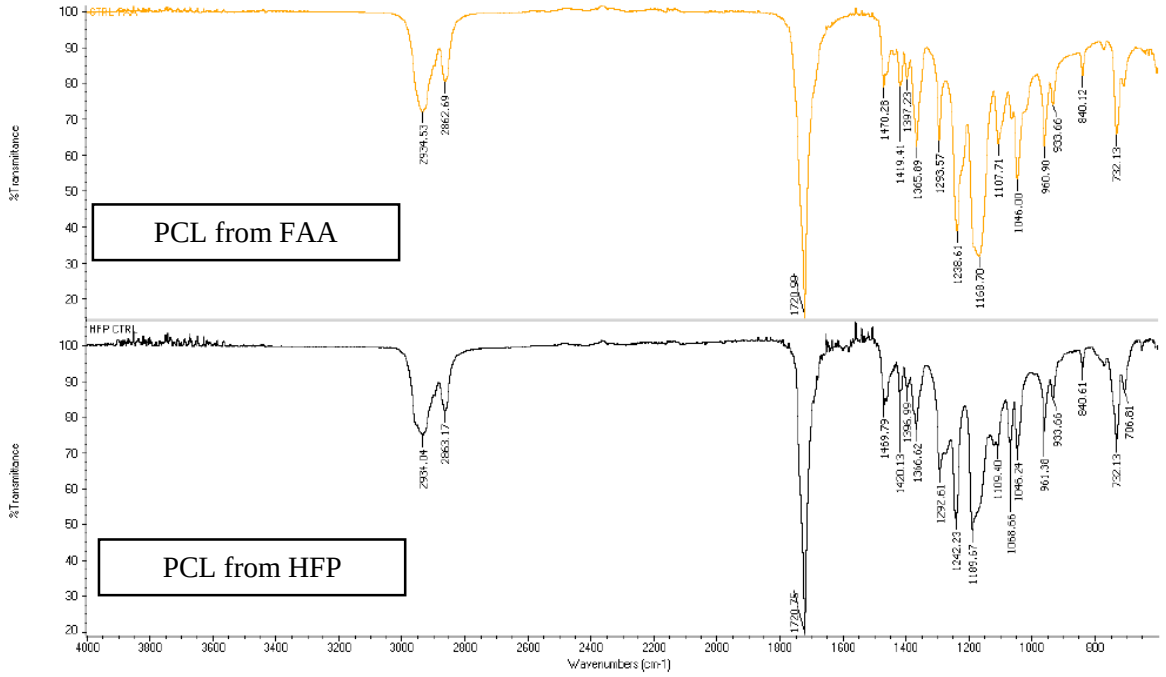
a. Characteristics infrared bands of PCL

Position (cm^{-1})	Vibrator	Abbreviation
2949	Asymmetric CH_2 stretching	$\nu_{as}(CH_2)$
2865	Symmetric CH_2 stretching	$\nu_{s}(CH_2)$
1727	Carbonyl stretching	$\nu(C{=}O)$
1293	C–O and C–C stretching in the crystalline phase	ν_{cr}
1240	Asymmetric COC stretching	$\nu_{as}(COC)$
1190	OC–O stretching	$\nu(OC{-}O)$
1170	Symmetric COC stretching	$\nu_{s}(COC)$
1157	C–O and C–C stretching in the amorphous phase	ν_{am}

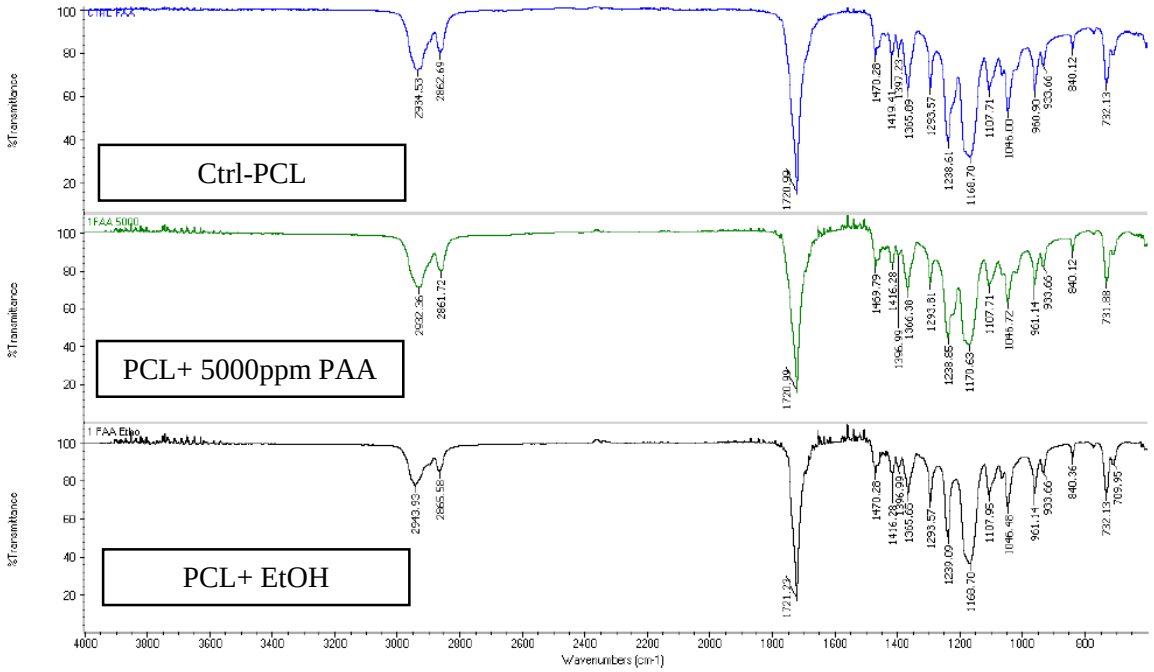
b. Effect of Peracetic Acid Concentration on Electrospun PCL



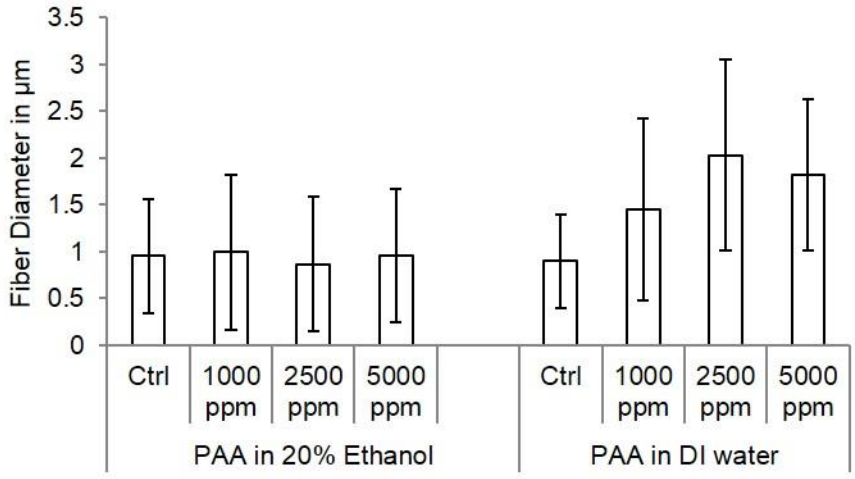
c. Influence of solvent used in electrospinning of PCL



d. Impact of Ethanol sterilization and Peracetic acid sterilization on PCL

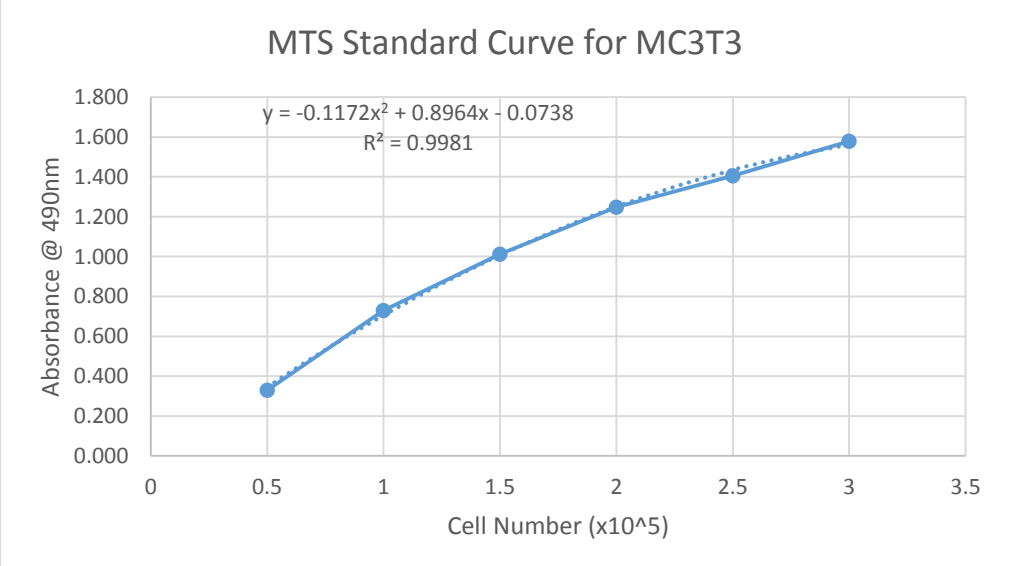


2) Evaluation of fiber diameter from scanning electron micrographs.



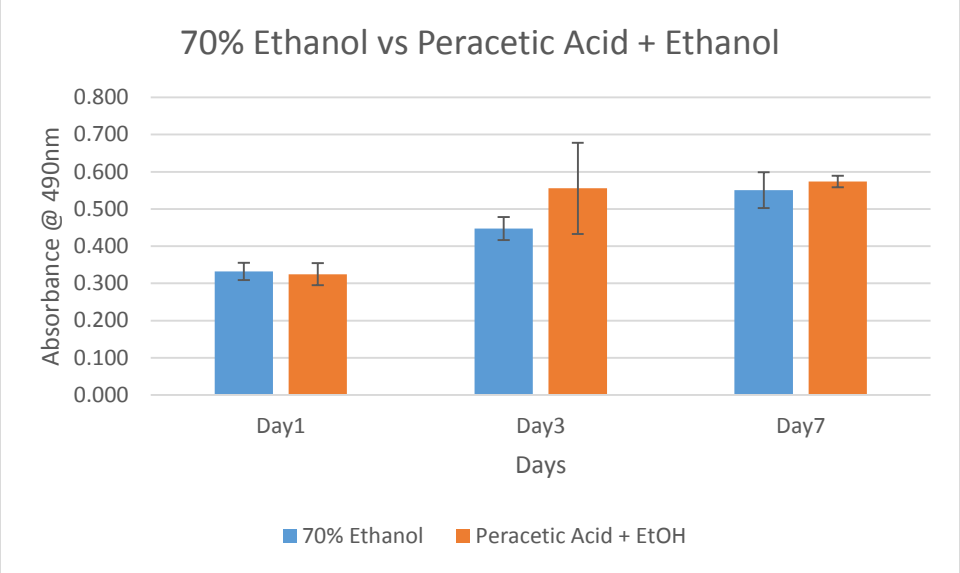
While the fibrous structure in scaffolds treated with PAA diluted in 20% ethanol is unaffected, scaffolds treated with PAA in water showed a trend towards increasing fiber diameters due to fusion of fibers.

3) MTS standard curve for MC3T3

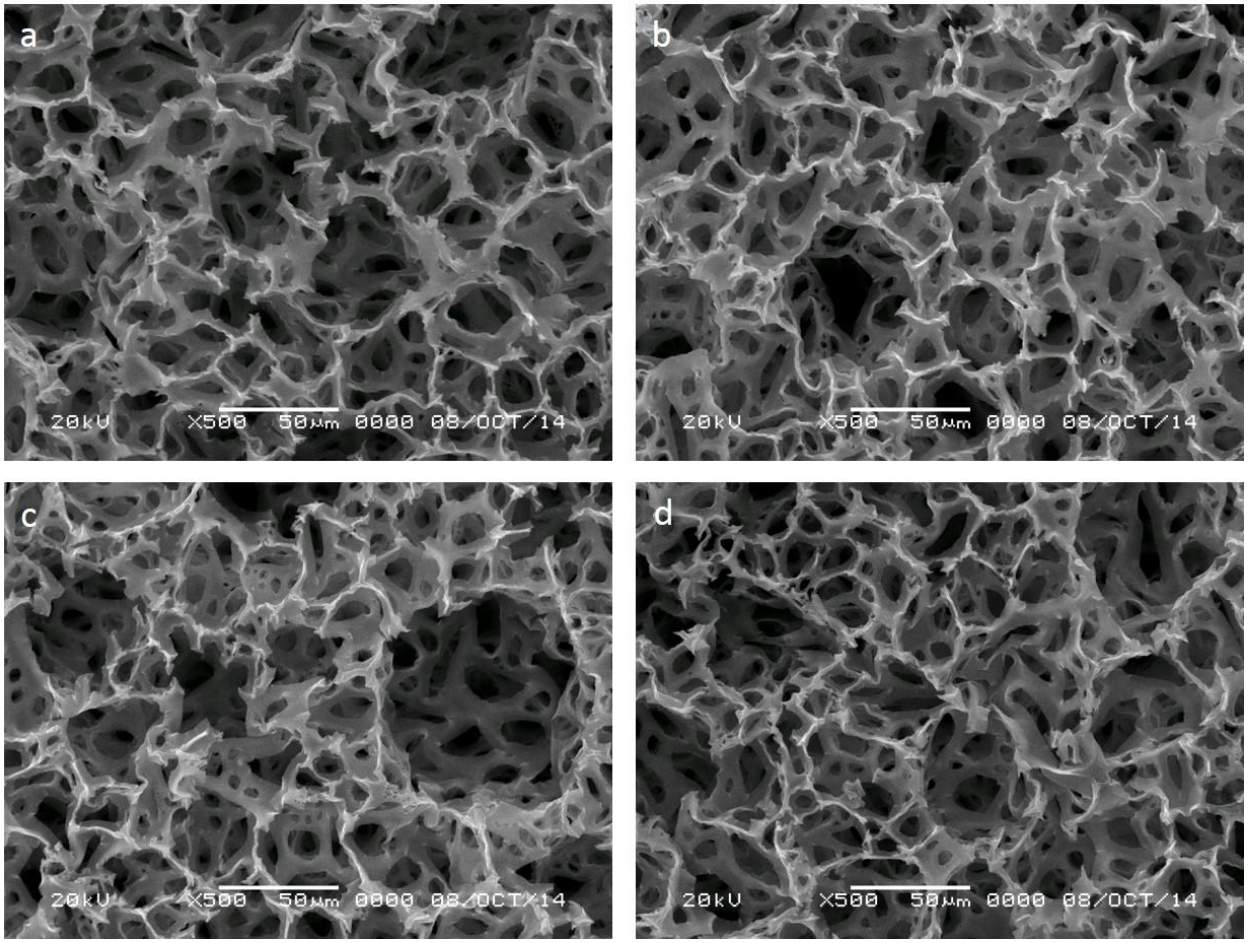


MC3T3 Cell Number x10^5	Average OD
0.5	0.329
1	0.729
1.5	1.013
2	1.249
2.5	1.405
3	1.578

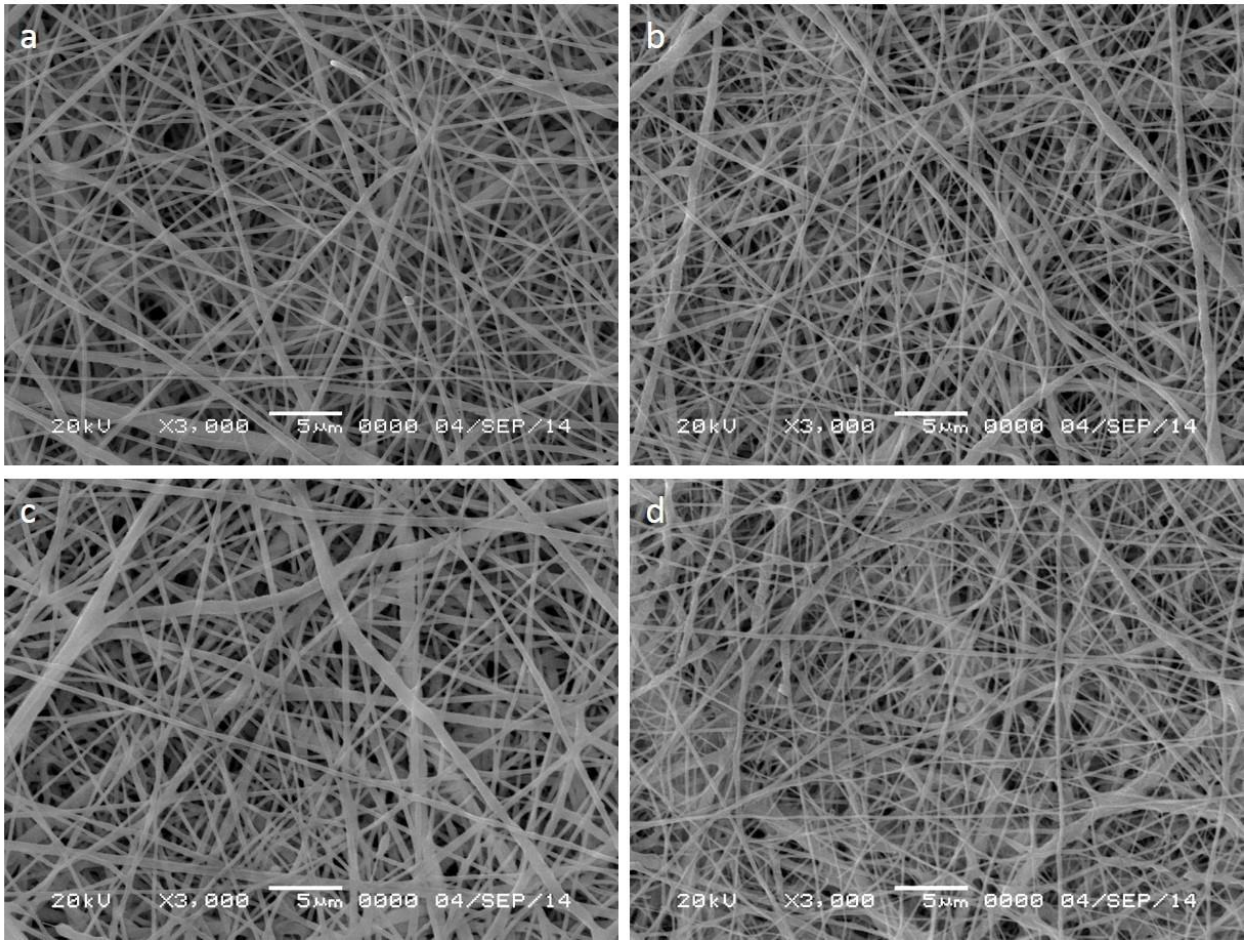
4) MC3T3 Cell Proliferation on Ctrl-PCL and PAA-PCL+EtOH on days 1, 3 and 7



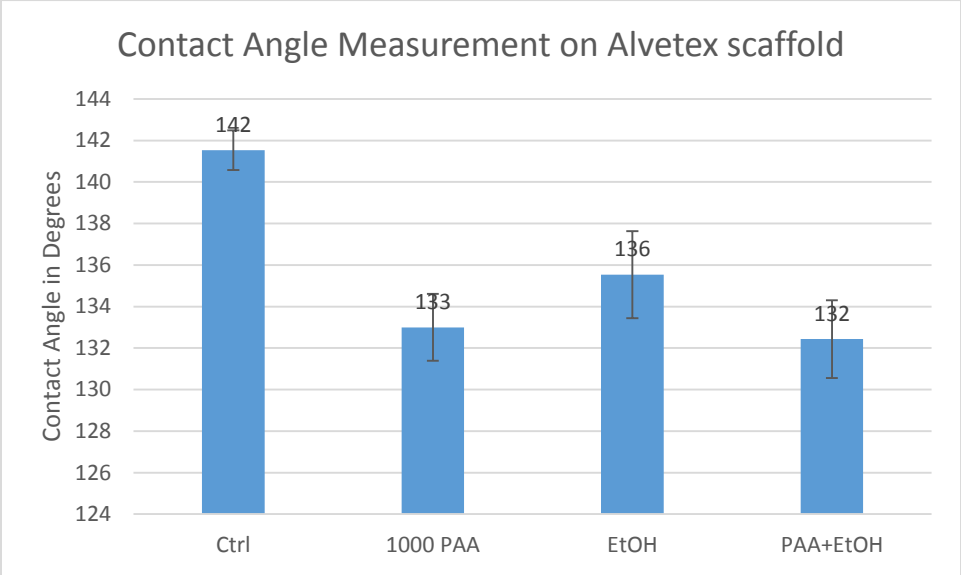
5) SEM of Alvetex Scaffolds exposed to different sterilization regimes **a)** Out of the box scaffold **b)** Ctrl-Alv (70% Ethanol) **c)** PAA-Alv **d)** PAA-Alv+EtOH



6) SEM of Electrospun PCL Scaffolds exposed to different sterilization regimes **a)** No treatment **b)** Ctrl-PCL (70% Ethanol) **c)** PAA-PCL **d)** PAA-PCL+EtOH



7) Contact angle measurements done on Alvetex scaffolds following different treatments.



VITA

Suyog Yoganarasimha was born on June 10, 1987, in Mysore India. He attended Sri Jayachamarajendra College Of Engineering and graduated in 2009 from Visvesvaraya Technological University with a Bachelor of Engineering degree in Biotechnology. After graduating with an engineering degree, Suyog worked for Oracle - Health Sciences Global Business Unit and was designing and implementing Siebel CTMS (Clinical Trial Management System) and OCDA (Oracle Clinical Data Analytics) applications. In 2013 he matriculated at Virginia Commonwealth University to pursue his graduate education in Biomedical Engineering.