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# Biodegradable polymer-bioactive ceramic composites for Guided Bone Regeneration

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**Biodegradable polymer-bioactive ceramic composites for Guided Bone Regeneration**

by

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## DEDICATION

I would like to dedicate this work to all the researchers who are working extremely hard to improve the quality of life of people suffering from terrible medical conditions.

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## NOMENCLATURE

| | |
|---|---|
| AM | Acetoxymethyl |
| ANOVA | Analysis of Variance |
| BF | Brightfield |
| BTE | Bone Tissue Engineering |
| $CaCO_3$ | Calcium Carbonate |
| CaP | Calcium Phosphate (Biphasic) |
| CTB® | CellTiter-Blue® |
| CS | Chitosan |
| CARS | Coherent anti-Stokes Raman Spectroscopy |
| CI | Confidence Interval |
| CPS | Cycles per Second |
| CPLA | Copolymerized poly (lactic acid) |
| DCM | Dichloromethane |
| DSC | Differential Scanning Calorimetry |
| DMF | Dimethylformamide |
| DMEM | Dulbecco's Modified Eagle's Medium |
| EDC | 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide |
| EthD-1 | Ethidium Homodimer-1 |
| E-PTFE | Expanded High-Density Polytetrafluoroethylene |
| ECM | Extra Cellular Matrix |
| FBS | Fetal Bovine Serum |
| GTA | Glutaraldehyde Treatment |

| | |
|---|---|
| GBR | Guided Bone Regeneration |
| GTR | Guided Tissue Regeneration |
| HSD | Honestly Significant Difference |
| HA | Hydroxyapatite |
| iMED | Interdisciplinary Manufacturing and Design Engineering Lab |
| ISU | Iowa State University |
| MWNTs | Multiwalled Carbon Nanotubes |
| NHANES | National Health and Nutrition Examination Survey |
| NIDCR | National Institute of Dental and Craniofacial Research |
| NHS | N-Hydroxylsuccinimide |
| NMP | N-Methyl-2-pyrrolidone |
| PA | Peptide Amphiphiles |
| PBS | Phosphate-Buffered Saline |
| PCL | Polycaprolactone |
| PDLLA | Poly (D, L-lactic acid) |
| PEG | Polyethylene Glycol |
| PEO | Polyethylene Oxide |
| PGA | Poly (glycolic acid) |
| PLLA | Poly (L-lactic acid) |
| PLA | Poly (lactic acid) |
| PLGA | Poly (lactic-co-glycolic acid) |
| PVA | Polyvinyl Alcohol |
| RANK-L | Receptor Activator of Nuclear Factor Kappa-B Ligand |

| | |
|---|---|
| SEM | Scanning Electron Microscopy |
| SF | Silk Fibroin |
| NaCl | Sodium Chloride |
| SD | Standard Deviation |
| Ti-d-PTFE | Titanium Reinforced High-Density Polytetrafluoroethylene |
| TCP | Tricalcium Phosphate |
| TFA | Trifluoroacetic Acid |
| TPEF | Two Photon Emission Fluorescence |
| US | United States |
| XRD | X-Ray Diffraction |

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## ABSTRACT

This study aims to validate the preparation approach of novel biodegradable polymer-bioactive ceramic composites of poly (lactic acid) (PLA), chitosan (CS), and tricalcium phosphate (TCP) and evaluate their suitability for Guided Bone Regeneration (GBR). GBR is a dental surgical procedure that uses barrier membranes to direct the growth of new bone tissues. Resorbable membranes eliminate the need for a second surgery that patients with non-resorbable membranes need. Cryomilling, a solid-state, low-temperature blending process, facilitates bulk fabrication by eliminating difficulties such as high viscosity, insolubility and long processing times. It is a cost-effective technique to generate particles with high surface area to volume ratio, which provide a larger area for biological activity. Electrospinning was used to fabricate fibrous barrier membranes using the biocomposites prepared by cryomilling. X-Ray Diffraction (XRD) and Differential Scanning Calorimetry (DSC) were utilized to characterize the molecular structures, identify the glass transition and melting temperatures and to confirm the occurrence of homogeneous polymer-ceramic biocomposites. Scanning Electron Microscopy (SEM) was used to observe the morphology of the powder composites and the electrospun membranes. RAW 264.7 murine macrophages were used to evaluate the cytocompatibility of the biocomposites and quantitatively analyzed with CellTiter-Blue® (CTB®) cell viability assay. Also, MG63 cells were seeded on electrospun membranes to quantify the capability of the biocomposites to encourage cell proliferation. Coherent anti-Stokes Raman Spectroscopy (CARS) and brightfield (BF) microscopy were used to analyze cell proliferation on the seeded membranes qualitatively. A 21-day *In vitro* degradation studies were performed and analyzed using Raman spectroscopy. CTB® cell viability assay carried out on the electrospun membranes revealed that the cells are viable and

metabolically active both at 3 and at 7 days from cell seeding indicating the suitability of the material for GBR.

# CHAPTER I

# GENERAL INTRODUCTION

## 1.1. Research Motivation

In the recent years, the increasing interest in developing dental procedures for bone regeneration has been a consequence of the growing number of patients in need of such interventions. Based on the data from the 2009 and 2010 National Health and Nutrition Examination Survey (NHANES), Eke et al. (2012) reported that 64.7 million adults in the United States (U.S.) suffer from periodontitis with varying levels of severity [1]. The results of similar surveys only reiterate the prevalence of periodontitis and have rightly warranted attention from the scientific community [2-4].

Periodontitis is a dental disease that is characterized by the destruction of the connective tissue and dental bone support due to an inflammatory response to the infection caused by the activity of anaerobic bacteria [5, 6]. Today, periodontists use a wide variety of techniques to treat this condition depending on the tissue affected and the severity of damage caused to the tissues. Some of the common methods used are laser treatment, gum graft surgery, non-surgical treatment such as scaling and root planing, dental crown lengthening, pocket reduction procedures and dental implants [7]. However, over the past few decades, regenerative techniques have received significant attention for restoring the functionality and structural integrity of a diseased periodontium, as it could potentially solve problems such as shortage of bone grafts and graft rejection [8-13].

Guided Bone Regeneration (GBR) is a surgical technique in dentistry that makes use of barrier membranes to exploit the inherent regenerative capability of the human body [14]. GBR has been able to treat various bone defects caused by periodontitis [15]. In principle, GBR uses barrier

membranes to prevent the entry and proliferation of non-osteogenic cells into defect sites and to permit the growth of osteogenic cells selectively [16]. Therefore, the success of the procedure, amongst other factors, is also largely governed by the design and performance of the barrier membrane. A suitable membrane has to be biocompatible, mechanically stable and flexible during the time of implantation [17].

The engineering community has been particularly interested in the development of new materials that could be utilized for the fabrication of barrier membranes. The number of scientific papers focusing on the production and evaluation of new membrane materials is a testament to the potential in this area [18-22]. Despite this, the "ideal" membrane has not yet been fabricated. Expanded polytetrafluoroethylene (e-PTFE) has by far been the most preferred material for the fabrication of non-resorbable membranes [23-30]. However, it has been shown that patients are exposed to infections when they undergo surgeries for the removal of non-resorbable membranes [31, 32]. Resorbable membranes have played the role of alternatives by eliminating the need for removal surgeries [33]. Natural polymers like chitosan, collagen and synthetic polymers like poly (lactic acid) (PLA), polycaprolactone (PCL), poly (lactic-co-glycolic acid) (PLGA) have been used individually or in blends for the fabrication of barrier membranes [34-44]. In the recent years, the addition of bioactive ceramics has been used to improve the mechanical properties and cell affinity of barrier membranes [45, 46]. Composite materials have thus offered researchers an opportunity to fabricate tailor-made membranes for specific applications.

This study intends to validate the fabrication approach of novel biodegradable polymer-bioactive ceramic membranes for use in GBR. A low-temperature, solid-state, blending technique called cryomilling was used to generate composites made up of PLA, CS, and TCP. As mentioned previously, PLA has produced considerable success when used for GBR applications.

CS, apart from being resorbable and biocompatible, was also chosen for its antimicrobial properties [35]. The role of CS in cell adhesion, proliferation, and osteoblast differentiation has been well documented [36-38]. TCP was used as a synthetic bone substitute material to induce the regeneration of bone tissue [39-41]. PLA and CS have very different polarities and hence achieving a compatible blend of these materials pose a challenge [47]. Moreover, when TCP is dissolved in solvents for processing, rheological properties limit it's loading, with the aggregation and delamination occurring at high loading percentages [48]. This study validated the potential of cryomilling to overcome such limitations associated with the fabrication of biocomposites.

XRD was used to confirm the occurrence of a homogeneous composite powder blend. DSC was utilized for the thermal characterization of biocomposites prepared by cryomilling. The cytotoxicity of the biocomposites was evaluated using RAW 264.7 murine macrophages. The cell viability was investigated using CTB® cell viability assay at the end of 24 and 48 h from the time of cell seeding. The cell morphology was qualitatively evaluated using BF microscopy. The powder composites were then spun into nanoscale fibers using electrospinning. SEM was used to observe the morphology of the fibers generated. CTB® cell viability assay was used to evaluate the proliferation of MG63 cells on electrospun membranes quantitatively. A live/dead viability assay was conducted, and the fibers were qualitatively analyzed using CARS and fluorescence microscopy. Also, a 21-day *In vitro* degradation study was performed on the electrospun membranes and analyzed using Raman spectroscopy. All data are expressed as mean values along with minimum and maximum values. Statistical analysis was carried out using Tukey's post hoc test of two-way Analysis of Variance (ANOVA) with PRISM ver. 7.0 software. Values of $p<0.05$ were taken to indicate statistical significance.

In conclusion, the goal of this research study is to fabricate fibrous membranes made out of cryomilled PLA/CS/TCP biocomposites that will stimulate the regeneration of the alveolar bone tissue.

## THESIS ORGANIZATION

In this thesis, Chapter 1 presents the general introduction and information of this study. Chapter 2 provides the motivation behind this research by presenting significant background information on certain key topics. Chapter 3 intends to provide a brief summary of various observations that were made during the pilot studies, and it also presents the logical reasoning for the modifications made in the experimental design. Chapter 4 illustrates the detailed experiment design and implementation, together with results and conclusions about the suitability of the generated biocomposites for GBR. Chapter 5 provides general findings and future research directions. Appendix A describes briefly the statistical analyses that were performed to analyze the data obtained from in vitro studies.

# CHAPTER II

# REVIEW OF THE LITERATURE

This section intends to discuss the motivation behind this research by providing significant background information on certain key topics related to this study. The rationale behind the design and development of this research study has been provided along with inferences from important research works published on the subject of interest.

This section is divided into six parts: 1) Periodontitis, 2) Guided Bone Regeneration, 3) Materials for barrier membranes, 4) Fabrication of polymer blends, 5) Production of polymeric nanofibers, 6) Summary of literature

## 2.1. Periodontitis

### *2.1.1. Periodontitis*

Periodontitis is a dental disease that is characterized by the destruction of connective tissue and dental bone support due to an inflammatory response to the infection caused by bacteria (Fig. 1) [5, 6]. Periodontitis is a degenerative disease that starts off with a reversible condition known as gingivitis which is characterized by the inflammation of the gums. The continuous build-up of plaque and tartar is responsible for the formation of pockets between the gums and the teeth. These pockets act as breeding grounds for bacteria and cause the inflammation of the gums. When left untreated, gingivitis can progress to an advanced stage and cause periodontitis. Periodontitis, unlike

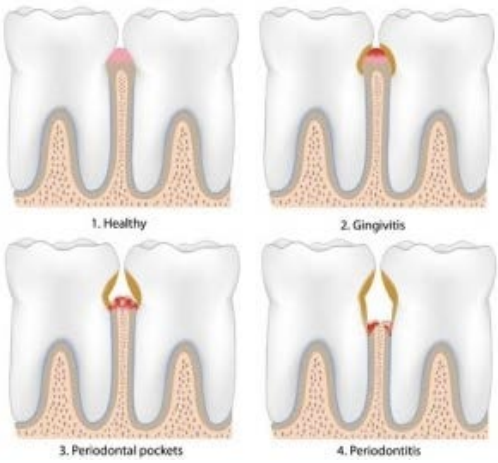


**Fig. 1.** Progression of periodontitis[1]

gingivitis, is an irreversible condition that leads to the destruction of periodontal tissues and, eventually, tooth loss [11].

*2.1.2. Prevalence of Periodontitis*

Currently, 5-20% of the adult population worldwide suffer from severe periodontitis which can ultimately result in tooth loss [49-51]. Based on the data from the 2009 and 2010 National Health and Nutrition Examination Survey (NHANES), Eke et al. (2012) reported that 64.7 million adults in the United States (U.S.) suffer from periodontitis with varying levels of severity [1]. It has also been understood that children and adolescents can also suffer from different forms of periodontitis ranging from aggressive to chronic periodontitis [52-54]. Moreover, the data from National Institute of Dental and Craniofacial Research (NIDCR, National Institutes of Health, U.S.) suggests that about 90% of adult populations more than 70 years old suffer from at least a moderate level of periodontal disease [2-4]. The high-level prevalence of periodontitis can be attributed to its association with several systemic disorders.

*2.1.3. Risk Factors*

Over the years, researchers have conducted numerous studies to understand the different factors that can influence the possibility of periodontitis. These risks have been broadly classified into two distinct categories namely modifiable and non-modifiable risk factors. The presence and accumulation of bacterial species have been identified as the major modifiable risk having a significant influence in increasing the risk of periodontitis. *Bacteroides forsythus*, *Prevotella intermedia*, *Peptostreptococcus micros* and *Fusobacterium nucleatum* have been strongly linked

to the progression of periodontitis in adults [55-58]. The other modifiable risks include smoking, diabetes mellitus, cardiovascular disease, stress, and obesity [59-69]. Osteoporosis, female hormonal alterations, pregnancy, host response, and age have all been identified as non-modifiable risk factors that have a significant correlation with periodontitis [70-73].

*2.1.4. Mechanism of Bone Resorption: Understanding Periodontitis*

Periodontitis affects the structural integrity of the periodontal tissues. The periodontium consists of the root cementum, alveolar bone, periodontal ligament and the dentogingivial junction (Fig. 2). Each of these components have individual and distinct roles to play to maintain the overall function of the periodontium. A person suffering from a periodontal disease will experience a progressive destruction of the periodontium. Researchers have also been always working on decoding the mechanism of bone resorption in periodontitis to design effective treatment techniques. It has now been widely accepted that the loss of alveolar bone is a natural consequence of the progression of periodontitis. The loss of the alveolar bone results in the formation of a pocket around the tooth which then acts as a reservoir supporting the growth of anaerobic bacteria which in turn leads to tooth loss [74]. After studying the mechanism of bone resorption, Heinz et al. (2015) concluded that "The Th1-type T lymphocytes, B cell macrophages, and neutrophils promote bone loss through upregulated production of proinflammatory mediators and activation of RANK-L expression pathways" [75]. This response disturbs the delicate balance between protective and destructive functions of the immune system [76-81].

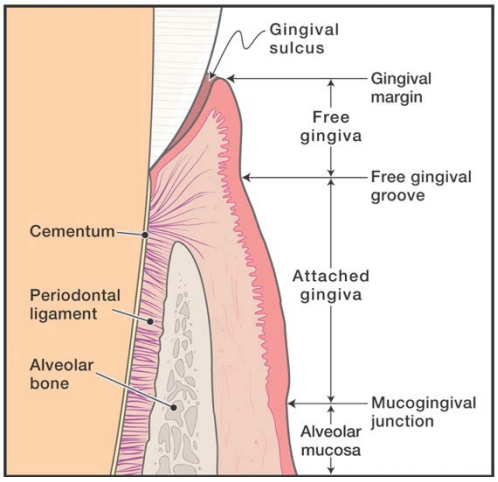


**Fig. 2.** Cross-sectional image of the periodontium[2]

*2.1.5. Treatment Options*

Periodontists use a wide variety of techniques to treat patients suffering from gingival recession and periodontitis depending on the severity of the condition. Some of the common methods used are laser treatment, gum graft surgery, non-surgical treatments, dental crown lengthening, pocket reduction procedures and placement of dental implants. Recently, engineers and periodontists have been particularly interested in regenerative processes that make use of barrier membranes to restore the functionality and structural integrity of a diseased periodontium. At this point, it is imperative to understand that regenerative procedures do not act as a treatment for periodontitis. Rather, it is an engineered approach for regenerating defects that have been caused by periodontitis. Therefore, appropriate periodontal treatment needs to be administered before regeneration is initiated [82].

In the recent years, the developments in the field of regenerative medicine have made engineers and clinicians believe that these procedures could be used to exploit the inherent regenerative capabilities of the human body. It is believed that regeneration techniques could solve the problem of shortage of bone grafts and challenges associated with graft rejection [12, 13, 83]. Therefore, in this particular study, the focus will be on regenerative surgical modalities that have been used to regenerate diseased tissues.

## 2.2. Guided Bone Regeneration

*2.2.1. Bone Augmentation*

Typically, patients with loss of teeth due to periodontitis need to undergo an implant therapy to fill an edentulous site. It is now widely accepted that the end goal of an implant therapy is to provide a completely functional implant that also caters to the aesthetic expectations of the person undergoing the treatment. However, a successful implant therapy demands an alveolar

ridge with appropriate dimensions to ensure success of the procedure. Researchers have concluded that tooth extraction can result in greater resorption leading to severe loss of alveolar width [84-86]. It has also been shown that, without treatment, post tooth-removal, crestal bone resorption is unavoidable 87, 88]. Therefore, it is important to have an intermediate step for the regeneration of alveolar deficient sites prior to implant placement. This need has necessitated the development of materials and techniques that can provide predictable and reliable results [89]. Researchers have developed and tested different treatment modalities such as ridge augmentation before implant placement, immediate implant placement in fresh extraction sockets, ridge augmentation with implant placement and ridge augmentation in combination with implant placement [90-99].

*2.2.2. Guided Bone Regeneration*

Guided Bone Regeneration treatment is based on the idea that alveolar and mandibular bone defects can be regenerated by using barrier membranes, which can mechanically isolate the defect site from non-osteogenic cells and selectively support the growth of osteogenic cell populations (Fig. 3) [14]. Approximately 60 years ago, the concept of secluding an anatomic site with the aim to promote healing was introduced, when cellulose acetate fibers were used to regenerate damaged nerves cells and tendons [100, 101]. Around the same time, in a different study, researchers reported enhanced wound healing of rib, femoral and radial bone defects by using barrier membranes [102, 103]. Later on, favorable results were reported by placing barrier membranes over jawbone defects in rabbits and cranial defects in rats [104, 105]. All these experimental studies reinforced the belief in the idea of GBR which was first

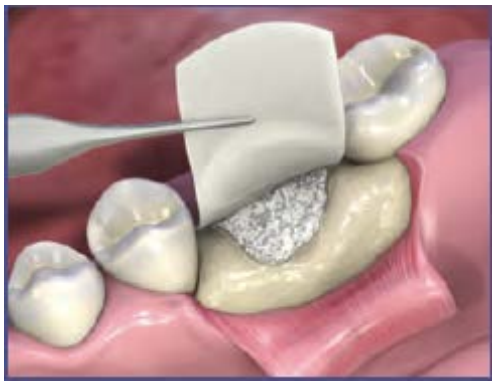

**Fig. 3.** Application of a barrier membrane[3]

introduced in a work published by Dahlin et al. (1988) [10]. Over the years, researchers have built on the foundation laid by these initial studies and have successfully used the concepts of material chemistry to develop efficient membranes. Currently, GBR is one of the most promising treatments available for patients to regain sufficient bone volume at the implant site [14, 106-108]. The occlusive membranes used in this treatment method promote the growth of new bone tissue by acting as a scaffold [109]. Clinically, it has been claimed that the barrier membranes used for the treatment should support new bone formation and maturation for at least six weeks [110, 111]. Hence, it is important to design and fabricate occlusive membranes with optimal persistence and stability to guarantee the success of the procedure.

*2.2.3. Barrier Membranes*

For the GBR technique, irrespective of the use of bone graft material, the barrier membrane has been proven to play a vital role in the prevention of epithelial tissue migration into the defect site, and in consequently allowing sufficient time for bone, cementum and ligament regeneration [112, 113]. The occlusive membranes are broadly classified into resorbable and non-resorbable membranes based on their ability to degrade inside the human body. The non-resorbable membranes made out of expanded polytetrafluoroethylene (e-PTFE) have been extensively studied and are still considered the gold standard in the industry [114]. However, in the recent years, researchers have shown tremendous interest in fabricating resorbable membranes because it eliminates the need for a removal surgery which is required in the case of their non-resorbable counterparts [11, 115]. The use of resorbable membranes has made the procedure less traumatic for the patients undergoing the treatment. Moreover, it has been suggested that the stiff non-resorbable membranes may result in soft dehiscence and subsequently progression of infection

[116]. However, as far as resorbable membranes are concerned, there are still challenges that need to be addressed such as rapid degradation of the membrane, lack of structural integrity and reduced osteoconductivity [117]. One of the significant challenges with the resorbable membrane has been to match its resorption time with the rate of tissue formation [118]. In conclusion, ideal barrier membranes for GBR need to exhibit: a) mechanical strength, b) biocompatibility, c) clinical manageability, d) osteoconductivity and preferably e) a degradation rate that matches the rate of bone tissue formation [11, 119-121]. Over the years, researchers have experimented with a wide variety of materials for the fabrication of GBR membranes.

## 2.3. Materials for Barrier Membranes

### *2.3.1. Materials for Non-resorbable Membranes*

The most regularly used materials to make non-resorbable membranes are expanded high-density polytetrafluoroethylene (e-PTFE) and titanium reinforced high-density polytetrafluoroethylene (Ti-d-PTFE) [122]. PTFE based membranes have been studied extensively and are especially noted for their excellent space-making ability [123]. A favorable correlation between space protection and bone regeneration has also been established [124]. These biocompatible and inert membranes are known to maintain their structural integrity. Amongst the two membrane materials, the titanium reinforced material has been shown to exhibit superior mechanical strength. Moreover, the increased mechanical strength allows the membrane to handle better the compressive forces exerted by the surrounding soft tissue and hence improves the regenerative capacity of the membrane [125]. The biggest drawback with non-resorbable membranes is that a second surgery is required to remove the membranes which implicate not only new pain but also economic discomfort [117].

*2.3.2. Natural Materials for Resorbable Membranes*

Biodegradable membranes are commonly made up of either natural or synthetic polymers. Amongst natural polymers, the commonly used ones are collagen and chitosan (CS). The other commonly used natural materials are gelatin and silk fibroin (SF). This section discusses each of these materials along with some of the important scientific observations that have been published.

*2.3.2.1. Collagen-based Membranes.* Collagen is a major component of the Extra Cellular Matrix (ECM) and hence has repeatedly been used due to its biocompatibility and good cell affinity [126-128]. Collagen membranes, majorly type I and type III, have favorable properties like fast vascularization, minimum immune response, good tissue integration, osteoblastic adhesion and a capability to promote wound healing [118, 129-134]. Despite having such favorable properties, the use of collagen-based membranes has been limited because of certain fundamental drawbacks. Firstly, Type-I collagen is considered to be expensive. Also, collagen-based membranes have demonstrated a poor space-making ability in humid conditions, inferior mechanical strength and they possess a degradation rate that is hard to control [135]. Additionally, it has also been suggested that these membranes have shown poor mechanical properties during their degradation [136]. It has been suggested that different cross-linking techniques can be used to improve the stability and biomechanical properties of collagen-based membranes [137-140]. Although techniques like cross-linking give certain incentives to use collagen for the fabrication of membranes, they introduce other problems such as prolonged integrity and reduced capability to cause angiogenesis [141-143]. Research also indicated that cross-linked membranes could cause adverse events and reduced bone regeneration in comparison to the non-crosslinked membranes [144]. Unlike

synthetic materials, collagen is derived from human or animal tissues. Hence, there is also an increased risk of infections.

*2.3.2.2. Chitosan-based Membranes.* CS is another natural polymer that has shown great potential to be used in the fabrication of barrier membranes. CS is a polysaccharide obtained by the deacetylation of chitin and has many applications due to its biochemical properties like antimicrobial effects, biodegradability, biocompatibility, and non-toxicity [145, 146]. CS is derived from shells of crustaceans, a natural resource and hence exhibits minimal foreign body response unlike polymers derived from mammalian proteins [147, 148]. It has been suggested that modified CS scaffolds demonstrate osteoconductivity in surgically created bone defects [149]. The antimicrobial and wound healing properties of CS has been attributed to its cationic nature [150]. Shin at al. (2005) suggested that the CS membranes were compatible with cells in in-vitro environments and also documented its role in bone regeneration [33]. Moreover, it has been proven that CS plays a supporting role in cell proliferation and osteoblast differentiation making it an attractive scaffold material for bone regeneration [43-45, 151]. However, the drawback of CS is its inferior mechanical properties which limit its use in load-bearing applications [152]. Over the years, researchers have found ways to work around this limitation. CS's attractive biomedical properties can be exploited by blending it with other synthetic polymers and ceramic materials to enhance its bioactivity and mechanical properties [153]. CS has been cross-linked with genipin to improve its immunogenicity and reduced mechanical properties. Researchers have reported faster healing time, superior mechanical properties and earlier infiltration timings with the use of such cross-linked CS membranes [154-156]. One of the significant contributing factors for the shift in attention towards CS is its low cost due to its large-scale availability in nature [157-160].

*2.3.2.3. Gelatin-based Membranes.* Gelatin is a soluble protein that is derived from partially denatured collagen. Just like chitosan, factors like availability and cost efficiency have encouraged researchers to use gelatin as a scaffold material [161]. Gelatin has been used in both guided tissue regeneration (GTR) and GBR applications owing to its attractive properties such as good cell adhesion, low immunogenicity, and biocompatibility [162]. However, fast degradation and poor mechanical properties have also been reported about gelatin. To combat these limitations, cross-linking with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), cross-linking with N-hydroxyl succinimide (NHS), glutaraldehyde treatment (GTA) and heat treatment has been used in the past [163-165]. It has been reported by Zhang et al. (2011) that the enhancement in elastic properties of gelatin is usually accompanied by a low Young's modulus hence limiting the use of gelatin in GBR and GTR membranes [163].

*2.3.2.4. Silk Fibroin-based Membranes.* SF is a natural protein that is extracted from silkworms or spiders, and it possesses properties that are expected of a scaffold material used in regenerative applications [166]. Some of its advantageous properties include excellent biocompatibility, oxygen permeability and biodegradability [167]. Kim et al. (2005) reported a complete union in calvarial defects of rabbits treated with SF based membranes. It was also said that SF provides incredible strength that improves the space-making ability of the scaffold [168]. In another independent study, the tensile strength of SF-based membranes was shown to be better than cross-linked collagen and PTFE membranes [169]. Even though there is considerable evidence that SF could be a potential candidate for the fabrication GBR scaffolds, it is slightly more expensive in comparison to CS. Moreover, SF demands a tedious preliminary processing before its use in biocomposite fabrication, unlike CS.

*2.3.3. Synthetic Materials for Resorbable Membranes*

In an attempt to overcome the inherent shortcomings of natural polymers, researchers have been working on using synthetic polymers in scaffold fabrication for regenerative medicine and drug delivery applications [98, 170-177]. The most commonly used synthetic polymers include polycaprolactone (PCL), poly (lactic acid) (PLA), poly (glycolic acid) (PGA) and poly (lactic-co-glycolic acid) (PLGA) [178-181]. Favorable properties like biocompatibility, biodegradability, clinical flexibility and commercial availability explain the increased usage of polyester-based membranes in tissue engineering applications [111, 135, 182].

*2.3.3.1. PLA-based Membranes.* PLA has been widely used for the fabrication of sutures, drug delivery systems, and scaffolds for tissue engineering [183, 184]. It has been suggested that PLA has been of great interest to biomedical and tissue engineers because of its hydrolysable ester bonds [185]. However, poly (L-lactic acid) (PLLA) has a degradation rate that is not favorable for the use in GBR. It takes about four years to degrade while the ideal membranes should be completely resorbed in under one year [186, 187]. To overcome this difficulty, researchers have often used copolymers of lactide and e-caprolactone, glycolide and other polymers to reduce the time taken for complete resorption. Vivosorb®, a commercially available membrane consisting of poly (D, L-lactide-e-caprolactone) was reported to be suitable for the purpose of regeneration with favorable properties like biocompatibility and non-cytotoxicity. Vivosorb® takes approximately 16 months to be completely resorbed [188]. Epi-Guide® is another resorbable, commercially available membrane consisting of poly (D, L-lactic acid) (PDLLA) with a unique three-layer technology that is known for its space-maintenance ability. Epi-Guide® maintains its structure and functions

for five months after implantation with a complete resorption in just over a year [189, 190]. Poly (lactic-co-glycolic-acid) has also been studied extensively in the past few decades [191].

*2.3.3.2. PLGA-based Membranes.* PLGA is similar to PLA regarding degradation mechanism where the hydrostatically unstable bonds hydrolyze into lactic acid and glycolic acid [192]. Resolut® is a commercially available product consisting of two layers where one layer prevents the growth of epithelial tissue while the other layer promotes the integration of tissues. Histological studies have shown that this product is as effective as non-resorbable membranes with regards to performance but with the additional benefit of being resorbable [193, 194]. Recently, Hua et al. (2014) have shown that PLGA based membranes can assist the formation of new bone trabeculae in beagle dogs [38]. The stiffness of PLGA membranes was a major problem until it was resolved with the addition of softeners like N-methyl-2-pyrrolidone (NMP). Some of the studies indicate that the addition of softeners could also accelerate the maturation of preosteoblastic cells and bone regeneration [195, 196]. The addition of lauric acid has also been suggested to improve the mechanical properties of pure PLGA membranes [197].

*2.3.3.3 PCL-based Membranes.* PCL has been an attractive option for tissue engineers trying to design scaffolds because of its low cost, high mechanical strength and excellent biocompatibility [198-200]. However, due to its slow resorption rate, its use in GBR membranes has been limited [18, 201, 203]. The benefit of using PCL lies in the fact that it does not produce an acidic environment during degradation. In spite of having decent properties, PCL is mostly blended or co-polymerized with other polymers before its use in scaffold fabrication.

*2.3.4. Ceramic Additives*

The primary goal of GBR is to use scaffolds to permit and promote the growth of the ECM and eventually support ossification. The presence of phosphate and calcium within the local environment is necessary to achieve this goal [203]. Ceramics such as calcium phosphate (biphasic) (CaP), TCP and hydroxyapatite (HA) have been widely employed in the scaffold fabrication process to make the scaffold resemble the mineral components of the human bone [109]. Other ceramics used for bone regeneration include bioactive glass, glass-ceramic, titanium and silica [153, 204-207]. The increasing use of bone substitute materials can be attributed to the inherent drawbacks of the autografting procedure such as donor site morbidity and limited availability of donor sites [208]. The different forms of calcium phosphates are used based on the crystal structure and dissolution rate required for that particular application [209]. Calcium phosphates have been shown to enhance osteoblast response thus improving the overall biological response to the fabricated scaffolds [210-212]. Researchers have successfully used different fabrication techniques such as electrospinning and electrospraying to add various forms of calcium phosphates to the scaffold to increase its bioactivity, adhesive and proliferative capabilities [37, 127, 213-218]. TCP has also been proven to be an ideal synthetic bone substitute material to induce the regeneration of bone tissue [219-221]. Koyama et al. (2004) showed that TCP increased bone regeneration 12 weeks after surgery [126]. Copolymerized PLA (CPLA) and TCP were prepared, and they were reported to have good biocompatibility with excellent mechanical properties [42]. Moreover, it provides calcium ions to the bone tissue to create a suitable ionic environment that will encourage bone formation [46, 222]. Jansen et al. (1995) fabricated HA-based composites and reported its excellent biocompatibility [223]. Even though the addition of ceramic additives has yielded scaffolds with better proliferative capabilities, ceramic addition has always been

limited to minuscule amounts due to processing and mechanical strength considerations [224, 225].

*2.3.5. Membranes based on Polymer Blends*

As GBR membranes require a broad variety of properties to succeed in regenerating the alveolar bone, it is almost impossible for a single material to be successful. For example, natural polymers almost always lack mechanical strength while synthetic polymers are not capable of inducing biological activity. It may be an efficient solution to blend different materials to hinder their drawbacks and showcase positive synergistic effects [18]. To combat the weak mechanical properties of PLGA, it was mixed with PCL in the same ratio, and their compressive strength and modulus were found out to be much higher than pure PLGA scaffolds [201]. It has also been suggested that PDDLA/PLGA composite scaffolds could serve as a barrier for tissue regeneration [202]. Besides the composites mentioned in this section, other synthetic blends may also have a bright future in GBR procedures [226, 227]. As far as natural polymers are concerned, a significant amount of research has been done to blend CS with other polymers to improve its mechanical and physical properties. For example, it was reported that the cell adhesion and proliferation of chitosan could be increased by blending it with gelatin [228]. Another study observed the effects of adding HA to the gelatin/CS membrane and reported that the membrane possessed sufficient mechanical and structural properties to be suitable for GBR [229]. On the other hand, natural polymers have also been blended with synthetic polymers to improve the properties of the synthetic polymers when used for GBR [230-234]. For example, PLLA/CS membranes have shown much better degradation characteristics and non-fibroblast penetration properties when compared to pure PLLA membranes [235]. Another study indicated that the incorporation of CS

into PCL-PEG membranes improved the collagen orientation of the regenerated periodontium [236]. Antibacterial agents and growth factors have been the other exciting additions to polymers [237].

Over the years, different techniques have used to blend materials together in order fabricate polymer blends and composite materials. However, the blending technique needs to be evaluated and chosen based on certain considerations such as materials to be blended, final application of the blend, processing time, economic feasibility, and, most importantly the efficiency of the process.

## 2.4. Fabrication of Polymer Blends

As mentioned in the previous sections, polymer-bioceramic composite scaffolds represent a convenient alternative for applications in hard tissue regeneration due to the possibility to tailor their various properties such as mechanical, structural behavior, degradation kinetics and bioactivity [238]. However, the inherent immiscibility of polymers and composites pose a challenge to the success of the material in bone tissue engineering (BTE) as the rheology of the dispersed phase might play a critical role in determining the manufacturability of scaffolds [239]. This section intends to discuss some of the compatibilization techniques commonly used in polymer processing.

*2.4.1. Melt Mixing with Block Copolymers*

Block polymers have repeatedly been used for the compatibilization of immiscible polymer blends, and their success has been well documented [240-242]. It has been shown that block polymers are thermodynamically favored to bridge the interface of immiscible polymer blends (Fig. 4). A few other kinds of polymers such as alternating, graft and gradient copolymers have also been used for similar applications. However, their usage remains limited as result of reduced commercial availability [243, 244]. Block copolymers have been observed to compatabilize immiscible blends by increasing the steric hindrance of domain motion and decreasing surface tension at the interface [245]. It has also been shown that larger copolymers exhibit improved compatibilization properties due to increased entanglement in both domains [246]. In general, it is believed that 5% of the dispersed phase interface must be saturated with block copolymer to prevent dynamic coalescence, while 20% would impart static stability [247]. Even though the success of this technique has been well documented, there have been issues that have repeatedly been reported about block copolymer compatibilization. One problem that has been reported in the literature is the self-assembling behavior of copolymers into micelles within the matrix phase [247]. Additionally, it has been reported that these micellar structures act as a contaminant and ultimately reduce the degree of compatibilization [248]. Another problem has been the necessary compromise that has to be made between using larger, efficient copolymers and diffusion problems associated with larger molecules. These issues are overcome by generating block copolymers within materials during processing at the blend interface [246]. However, this technique also has limitations such as

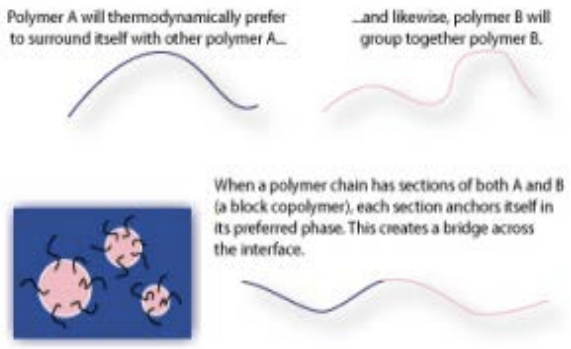


**Fig. 4.** Use of block copolymers in stabilizing the interface[4]

increased operating costs and slow reaction kinetics [249]. Moreover, this method is usually employed during melt mixing which uses high temperatures which could result in the denaturing of bioactive molecules [250].

### *2.4.2. Common Solvent Method*

In this technique, two incompatible polymers are dissolved in a common solvent, either under ambient or elevated pressures and temperatures. After complete dissolution, the solvent is removed by freeze drying or sublimation [251]. Unfortunately, this technique has several limitations and challenges, especially in the tissue engineering application. Homogeneous blending is a primary challenge during high loadings of filler materials [252]. The common solvent needs to be identified diligently as the incomplete dissolution of the polymers can reduce the efficiency of the process [253]. The use of toxic solvents has been shown to affect the biocompatibility of the materials involved [239]. The limitations mentioned above necessitate the development and use of low-temperature, solid-state techniques for the processing of biocomposites.

### *2.4.3. Cryomilling*

Cryomilling is a novel technique for alloying, but its advantages as a biomaterial fabrication method have yet to be leveraged on in the area of biocomposite fabrication [250]. It is the mechanical attrition of particles under a cryogenic environment. In material processing, it has often been used as a technique to strengthen the materials through grain refinement and dispersion of fine, nanoscale particles, mostly employed in the creation of dispersed metallic phases [254,

255]. Zhu et al. (2006) prepared polyaniline/iron composites using cryomilling [253]. Hou et al. (2012) have reported the fabrication and characterization of metal/ceramic powders [256]. However, in the recent years, researchers have used cryomilling to blend immiscible materials and generate mixtures which otherwise would have been difficult to produce [48]. As mentioned in the previous sections, there is a need to combine synthetic polymers with other materials like natural polymers and bioactive ceramics to elicit desirable biological responses from the human body. However, the blending of these materials is mostly achieved by dispersing the bioactive ingredients as the second phase in polymers solutions or melts [238, 239, 257, 258]. Some of the drawbacks to these traditional techniques include the phase separation of additives during blending which ultimately results in an inefficient blend. Also, it has been suggested by Coroller et al. (2013) that the homogeneity of dispersion is crucial to attaining superior mechanical performances from composite materials [259]. Polymer powder processing has previously been used for fabrication of pharmaceutical coatings and scaffolds for tissue engineering [260, 261]. Cryomilling has been used to create homogeneous blends of polymeric materials for the preparation of tissue engineering scaffolds [252]. Another study has shown that cryomilling can be utilized for the fabrication of biodegradable PCL/PGA scaffolds [262]. Apart from assisting with the manufacture of homogeneous blends, cryomilling also offers other benefits that cannot be expected of other traditional processing techniques. Cryomilling provides the benefit of producing a finer grain structure in relatively shorter processing times [263]. Researchers have found that particles with high surface area to volume ratio provides a larger area for biological activity [264]. Recently, Lim et al. (2014) fabricated PCL/TCP composite powders by cryomilling for tissue engineering and reported that cryomilling was able to achieve homogeneous dispersion even at higher loading percentages of ceramic particles [48]. Moreover, cryomilling requires lesser energy to induce a

material fracture when compared to other milling techniques as the whole process takes place below the glass-transition temperatures of the polymers used. In addition to all the advantages that have been previously mentioned, cryomilling is one method that can be used to overcome the creep behavior faced by polymeric scaffolds. By incorporating nanomaterials, it has been postulated to extend the viscoelastic response, resulting in high resistance to time-dependent strain results. Hence, it has been considered that the future of polymer biocomposites for utility in scaffold-based BTE lies in achieving homogeneous blends with exceptional mechanical properties [239].

## 2.5. Production of Polymeric Nanofibers

Tissue engineers have made extensive use of polymeric nanofibers for the purpose of tissue regeneration. The popularity of nanofibers can be understood by taking a look at the number of reviews focusing on their production, application, and interaction with biological cells [265-269]. The unique properties of polymeric membranes have made them an indispensable tool in the armory of a tissue engineer. The small diameter of these fibers closely matches the size and morphology of the ECM fibers. In general, electrospun fibers are used as biomimetic scaffolds, and their high surface area to volume ratio have only added to their advantages in the field of drug loading and regenerative medicine [270-272]. These unique properties have been extremely useful in modulating cell behavior [273]. Three production techniques are commonly employed for the fabrication of nanofibers in the field of tissue engineering. This section will briefly review the literature on electrospinning, phase separation and self-assembly.

*2.5.1. Phase Separation*

The phase separation method works by inducing the phase separation of a polymer solution into a polymer-rich and a poor polymer phase. It has been widely used for the fabrication of porous scaffolds for tissue engineering applications [274, 275]. In recent years, aliphatic polyesters have been used in this technique. Briefly, a polymer such as PLLA is dissolved in a suitable solvent and rapidly cooled to induce phase separation. Afterward, the solvent is exchanged with water, and the construct is subjected to freeze drying. Some of the variations in the process include altering the gelling temperature and changing the polymer concentration [276, 277]. Apart from aliphatic polyesters like PLLA and PLGA, this technique has also been extended to polyhydroxyalkanoate, CS, gelatin and also gelatin/apatite composites [278]. However, this process has still been limited to just a few polymers and also the difficulty of scaling up has restricted the usage of this technique [265, 277].

*2.5.2. Self-assembly*

Self-assembly is a bottom-up approach to nanofiber production that relies on weak noncovalent interactions to build nanofibers from smaller molecules [271]. The building blocks are either naturally occurring or designed specifically for the occasion [279]. Peptide-amphiphiles (PAs) are one of the commonly used building blocks and have been in existence for over a decade now [280]. The chemical structure of PAs permits the initiation of an assembly with just an adjustment in the ion content of the PA solution [281]. While this approach can generate nanofibers of the smallest scale, the processing procedure is still challenging and is limited to a small set of polymers. Also, this process can only create short fibers that are only a few microns long [264].

*2.5.3. Electrospinning*

Electrospinning is a cost-effective technique that exploits the principles of electrostatic forces to generate fibers that have diameters either in microns or the nano-scale. In a typical process, a polymer solution is extruded out of a syringe and aimed at a collector plate. A potential difference is applied between the needle and the collector plate which helps the polymer droplet overcome the surface tension and take the form of fibers with the solvent evaporating before it reaches the collector plate. Some of the factors that are commonly varied to produce fibers with the right diameter are polymer concentration, needle tip to collector plate difference, applied potential difference, the geometry of the collector plate and polymer flow rate [265, 267, 282-285]. A variety of polymers has been used for electrospinning. Some of the synthetic polymers include PCL, PLLA, polyurethane, copolymers of PEG and PCL [286- 290]. Additionally, composite fibers have also been produced with natural, synthetic polymers and bioceramic constituents [224, 291]. Researchers have used electrospinning previously for the fabrication of scaffolds for bone tissue engineering (BTE). For example, PLA/HA composite membranes were fabricated, and osteoblast cell adhesion was evaluated to be much better than that of pure PLA [292, 293]. To combat the problem of inefficient dispersion of ceramic particles, Kim et al. (2006) investigated the influence of a surfactant [294]. Mei et al. (2007) fabricated membranes for guided tissue regeneration (GTR) using PLA, Multiwalled Carbon Nanotubes (MWNTs) and HA and reported enhanced adhesion and mechanical strength [295]. Schneider et al. (2008) fabricated PLGA/TCP nanocomposite fibers and concluded that the membranes have a bright future in the regeneration of bone defects [296]. Recently, a layer by layer approach was used to fabricate PCL/$CaCO_3$ membranes [117]. Authors have also reported the difficulty experienced during the electrospinning of pure natural polymers and have often overcome this problem by adding synthetic polymers for

easier processing [297]. Yang et al. (2008) fabricated CS/polyvinyl alcohol (PVA) with HA biocomposite nano-scaffolds using electrospinning and concluded that the increase in HA content above 5% decreased the ultimate tensile strength and strain in failure. The scaffold's capability to enhance cell adhesion was also reported [298]. Zhang et al. (2008) fabricated HA/CS nanofibers and highlighted the great potential of using these fibers for BTE applications [299]. Badami et al. (2006) made PDLLA, PLLA, PEG-PDLLA and PEG-PLLA nanofibers to evaluate the influence of surface topography on the cell morphology and cell proliferation [300]. Hu et al. (2013) concluded that electrospinning with dip coating is a possible technology for producing membranes for GBR and GTR [301].

## 2.6. Summary of Literature

This literature review section is aimed to provide readers with a summary of the work done in the field of BTE focusing mainly on the regeneration of bone tissues. Key takeaways from the literature review can be summarized as follows (1) The design and fabrication of barrier membranes is crucial role in the success for GBR; (2) Composite materials have the potential to be the best option for barrier membranes; (3) Homogeneous dispersion of bioceramic particles is a key factor in electrospinning; (4) Cryomilling can be a cheaper and efficient alternative to traditional blending techniques.

# CHAPTER III

# PRELIMINARY WORK

Before the investigation of the primary hypothesis, pilot studies were conducted to understand the processing of polymer-ceramic composites. Two different processing techniques, electrospinning, and compression molding were initially pursued for the fabrication of GBR membranes. However, in the end, observations from the pilot studies, inferences from literature, and preliminary results from the *In vitro* studies had a significant influence on the way the experiments were finally designed. This section provides a brief summary of the various observations that were made during the pilot studies, and it also presents the logical reasoning for the modifications made in the experimental design.

## 3.1. Design and Fabrication of Biocomposites

### *3.1.1. Initial Composite Design*

A detailed literature review clearly revealed that the ideal material for the manufacture of barrier membranes had to be a blend of different materials [18]. In other words, the choice to generate composites for scaffolds was straight-forward. However, the constituents of the composites had to be chosen with care. To regenerate the alveolar bone which is an organic-inorganic composite, it would be ideal for the scaffold to be made of both these components. PLA was chosen to be the first polymer representative based on its previous success in regenerative applications [183, 184]. CS was an obvious choice to be the natural polymer-agent because of its superiority to collagen. Some of the beneficial properties that CS offers include improved biocompatibility, enhanced cell adhesion, the ability to support osteoblast proliferation, minimal

immune response, low cost and also antimicrobial properties [43-45, 145, 146, 150, 151]. As mentioned in section 2.3.4, the addition of bioactive ceramics has shown to improve the regenerative capability of barrier membranes. The usage of TCP in the fabrication of membranes for GBR is well-documented and has been met with considerable success [219-221]. So, TCP was chosen to be the bioceramic in the blend. Table 1 shows the different combinations in which the materials were generated in the pilot study. The intention behind the design of composites was to evaluate various combinations to identify suitable blends for scaffold fabrication. The combinations A0*, A1*, A2* and A3* were designed to understand the interaction between PLA and TCP without CS. The samples B0* through C3* were designed to study the interaction of all the three materials. Samples B0*-B3* had a relatively higher percentage of PLA in the polymer matrix while samples C0*-C3* had a greater percentage of CS. Finally, the samples D0*-D3* were designed to understand the interaction of CS and TCP in the absence of the binder material, PLA. In this study, anything more than 20% of TCP in the polymer matrix was considered to be high as previous studies have reported agglomeration of TCP particles at such percentages [48].

Later, electrospinning and compression molding were used to fabricate scaffolds with the biocomposites prepared by cryomilling.

**Table 1.** Material compositions used for the pilot study

| Sample | PLA/CS (Polymer Matrix) | TCP/Matrix |
|---|---|---|
| A0* | 100/0 | 0/100 |
| A1* | 100/0 | 10/90 |
| A2* | 100/0 | 20/80 |
| A3* | 100/0 | 30/70 |
| B0* | 70/30 | 0/100 |
| B1* | 70/30 | 10/90 |

**Table 1** continued

| Sample | PLA/CS (Polymer Matrix) | TCP/Matrix |
|---|---|---|
| B2* | 70/30 | 20/80 |
| B3* | 70/30 | 30/70 |
| C0* | 30/70 | 0/100 |
| C1* | 30/70 | 10/90 |
| C2* | 30/70 | 20/80 |
| C3* | 30/70 | 30/70 |
| D0* | 0/100 | 0/100 |
| D1* | 0/100 | 10/90 |
| D2* | 0/100 | 20/80 |
| D3* | 0/100 | 30/70 |

*3.1.2. Cryomilling*

Once the material compositions were finalized, the literature was reviewed to identify the ideal blending technique for the fabrication of biocomposites. The literature review revealed the advantages of cryomilling in biocomposite fabrication [239]. Some of the benefits of using cryomilling include use of reduced energy, homogeneous dispersion even at higher loading percentages of ceramic particles and the added benefit of being a solventless process [48]. Also, researchers from Interdisciplinary Manufacturing Engineering and Design Laboratory (iMED), Iowa State University (ISU), have tasted considerable success in fabricating polymer composites using cryomilling [250, 252, 253]. The second hurdle was to identify the process parameters to be employed in the cryomilling process. Based on observations made by Lim et al. (2013) and observations from trial experiments run at iMED, the total cryomilling time was chosen to be

20 mins [48]. The other process parameters such as precool time, cooling time, cycles per second (cps) and number of cycles were chosen based on the manufacturer's prescription.

## 3.2. Production of Membranes for Guided Bone Regeneration

After the blending and fabrication of biocomposites by cryomilling, electrospinning and compression molding were explored for the production of GBR membranes. The inferences from the experiments involving these techniques are provided in the following sections.

### *3.2.1. Electrospinning*

As nanofibrous scaffolds have had great success with GBR, electrospinning was an appropriate choice for the fabrication of membranes [292-296]. This study being the first to produce a three-material biocomposite powder blend for the fabrication of GBR membranes, only made it harder to identify the appropriate process parameters to ensure repeated fabrication of fibrous membranes. Moreover, the different material properties of the various constituents of the biocomposites was an added challenge. First attempts that were made to fabricate nanofibers used chloroform and dimethylformamide (DMF) (3:1 v/v) as the solvents to dissolve the composite blends based on the experiments reported by McCullen et al. for PLA/TCP composites [302]. Another solvent combination that was tried was dichloromethane (DCM) and DMF (7/3 v/v) [301]. However, both the solvent combinations mentioned above did not yield expected results with combinations that contained a high percentage of CS (C0*-D3*). This was attributed to CS's ionic character in dissolved state and three-dimensional networks of strong hydrogen bonds [47]. Also,

the composites with TCP posed a challenge because of their rheological properties which caused aggregation at higher loading percentages (A3*, B3*, C3*, D3*).

At the end of the pilot study, it was concluded that the high ceramic content in some cases and presence of CS in other cases were responsible for the failure of DCM, DMF, and chloroform in being the common solvent. It was also inferred that CS was insoluble at neutral and alkaline pH but was soluble in acidic media. Thus, trifluoroacetic acid (TFA) was chosen as the co-solvent for the purpose of electrospinning.

*3.2.2. Compression Molding*

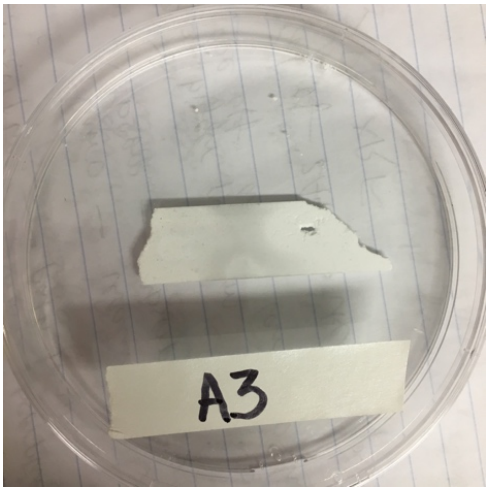


**Fig. 5.** A3* compression molded scaffold

Compression molding was used to fabricate bone tissue scaffolds using an entirely solvent-free approach [303]. Briefly, the aluminum mold was preheated to a temperature of 195°F, and a pressure of 5000 psi was applied for 15 mins once the composite material was placed inside. Fig. 5 is an image of the compression molded film that was fabricated using this protocol. However, the *In vitro* studies with MG63 cells did not yield expected reasons. Some of the scaffolds (A3*, B3*, C3*, D3*) crumbled when placed in the cell culture medium because of their relatively higher TCP content. Moreover, all the scaffolds lacked porosity and were not able to assist in guiding and promoting cell proliferation.

At the end of the *In vitro* study, it was inferred that a porous and interconnected scaffold architecture along with reduced TCP content would create an environment that would facilitate proliferation and migration of the cells.

## 3.3. Modified Experimental Design

### *3.3.1. Electrospinning*

The observations from work published by Tanase et al. (2014) were used to redesign the composites [185]. The biocomposites that were generated in this study are as follows: A0: 100% PLA; A1: 70% PLA, 30%CS; A2: 68% PLA, 30%CS, 2% TCP; A3: 66%PLA, 30%CS, 4%TCP. TFA was used as the co-solvent for the purpose of electrospinning. In a typical process, 18-20 w/v% of the composite powder was added to 5 ml of TFA, and the solution was stirred using a magnetic stirrer at 25 ℃ for at least 24 h for better dispersion and homogenization of the ceramic particles. The solution was then transferred to a 5 ml syringe with an 18G needle. A stationary copper plate covered with aluminum foil was used as the collector plate. The needle tip to collector distance was set to 13 cm, and the potential difference was adjusted between 15-17.5 kV as needed with a constant solution flow rate of 0.05 ml $min^{-1}$. Fig. 6 is a photograph of a barrier membrane that was prepared using electrospinning during this study. Fig. 7 is a micrograph of an electrospun membrane generated with A3.

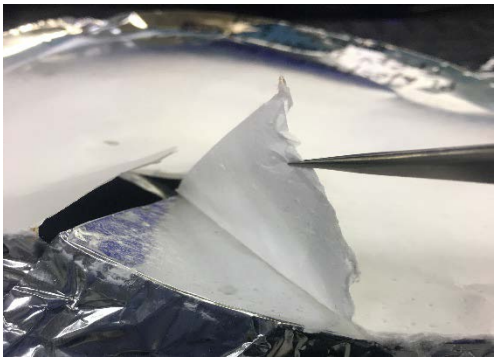

**Fig. 6.** Electrospun PLA/CS/TCP barrier membrane

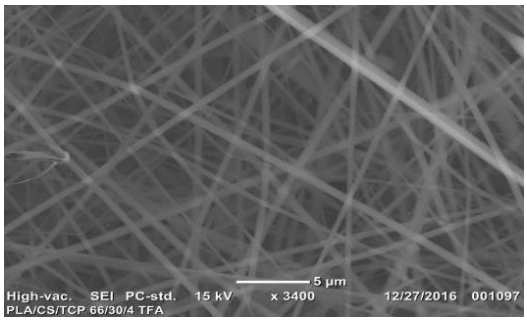


**Fig. 7.** Morphology of A3 membrane

### *3.3.2. Compression Molding*

The compression molding fabrication protocol was designed based on previously published work on BTE scaffolds [185, 304]. Compression molding was used to fabricate scaffolds, which

were later immersed in water for porogen leaching. Firstly, the composite powder and sodium chloride (NaCl) particles were mixed using a magnetic stirrer. The mass ratio of sodium chloride to composite powder was chosen to be 1.6:1 (w/w). After 30 mins of mixing, the mixture was transferred into a stainless-steel mold with cylindrical cavities (d = 9 mm; h = 13 mm). The compression molding process was carried out using a hydraulic press. The mold was preheated to 175℃, and the molding process was carried out with a pre-pressing step of 3 mins at 50 atm and a pressing step of 2 mins at 150 atm. The fabricated scaffolds were then porogen leached in distilled water for 24 h and dried in a programmable vacuum oven overnight. Fig. 8 shows a micrograph focused on the cross-section of the generated scaffolds. Fig. 9 indicates the dimensions and surface texture of the scaffolds that were generated. However, the *In vitro* studies once again revealed that the scaffolds underwent uncontrolled swelling in the culture medium and crumbled. So, the fabrication and the results of the compression molded scaffolds were omitted from the paper that has been submitted for peer-reviewed publication. Fig. 10 shows an image of the crumbled scaffolds in the culture medium. It was later hypothesized that the addition of a binder like polyethylene oxide (PEO) could improve the overall structural integrity of the scaffolds [305].

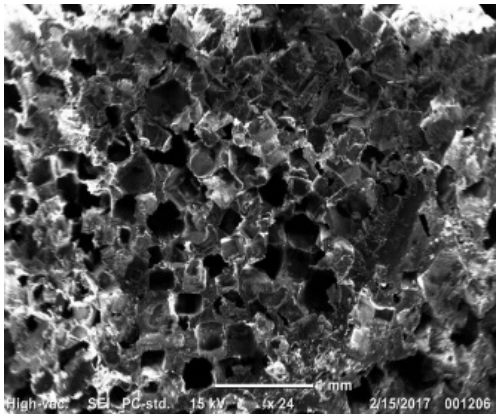


**Fig. 8.** Porous scaffold architecture (A2)

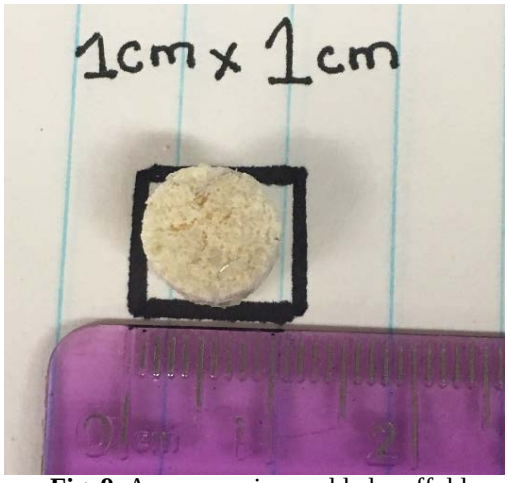


**Fig. 9.** A compression molded scaffold

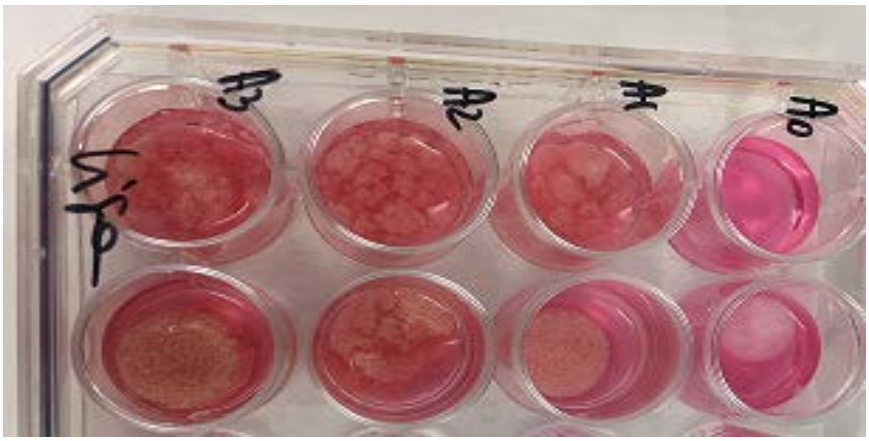


**Fig. 10.** Crumbled scaffolds in culture medium

# CHAPTER IV

## Fabrication and Evaluation of Poly (lactic acid)/Chitosan/Tricalcium Phosphate Biocomposites for Guided Bone Regeneration

Manuscript to be submitted to Materials Science and Engineering C

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### 4.1. Abstract

This study aims to validate the preparation approach of biodegradable polymer-bioactive ceramic composites, and evaluate their suitability for guided bone regeneration (GBR). GBR is a dental surgical procedure that uses barrier membranes to guide the growth of new bone tissues. Resorbable membranes eliminate the need for second surgeries that patients with non-resorbable membranes need. Cryomilling, a solid-state blending process, facilitates bulk fabrication by eliminating difficulties such as high viscosity, insolubility and long processing times.

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It is a cost-effective technique to generate particles with high surface area to volume ratio, which provide a larger area for biological activity. X-Ray diffraction and differential scanning calorimetry were used to characterize the molecular structures and glass-transition temperatures of the powder composites prepared by cryomilling. Scanning electron microscopy was used to study the morphology of the membranes generated by electrospinning. *In vitro* studies were performed with MG63 cells to quantify the blend's capability to encourage cell proliferation. Coherent anti-Stokes Raman spectroscopy and fluorescence microscopy were used to analyze cell proliferation qualitatively. A 21-day in vitro degradation study was performed on the electrospun membranes and analyzed using Raman spectroscopy. CellTiter-Blue® cell viability assay performed on cells grown on membranes revealed that cells are viable and metabolically active both at 3 and 7 days from cell seeding, indicating the suitability of the biocomposites for GBR.

*Key words: PLA/CS/TCP, Biocomposites, Guided Bone Regeneration, Cryomilling, Electrospinning*

## 4.2. Introduction

In the recent years, the increasing interest in developing dental procedures for bone regeneration has been a consequence of the growing number of patients in need of such interventions. Based on the data from the 2009 and 2010 National Health and Nutrition Examination Survey (NHANES), Eke et al. reported that 64.7 million adults in the United States (U.S.) suffer from periodontitis with varying levels of severity [1]. Periodontitis is a dental disease that is characterized by the destruction of the connective tissue and dental bone support due to an inflammatory response to the infection caused by the presence and activity of bacteria [2, 3]. Guided Bone Regeneration (GBR), when used, has been able to treat various bone defects caused by periodontitis [4-6]. In principle, GBR uses barrier membranes to prevent the entry and

proliferation of non-osteogenic cells in the defect sites by selectively permitting the growth of osteogenic cells [7]. Therefore, the success of the procedure, amongst other factors, is also largely dependent on the design and performance of the barrier membrane. A suitable barrier membrane is expected to be biocompatible, mechanically stable and flexible during the time of implantation [8]. It has been suggested that a barrier membrane used in GBR should support the formation and maturation of the new bone for at least six weeks [9-10]. Expanded polytetrafluoroethylene (e-PTFE) has by far been the most preferred material for the fabrication of non-resorbable membranes [11, 12]. However, it has been suggested that the use of stiff non-resorbable membranes may result in wound dehiscence exposing the operated site to infection [13]. Resorbable membranes have acted as alternatives by eliminating the need for removal surgeries [14]. Natural polymers such as chitosan (CS) [15-17], collagen [18] and synthetic polymers like poly (lactic acid) (PLA) [19-21], poly ($\varepsilon$-caprolactone) (PCL) [22-24], poly (lactic-co-glycolic acid) (PLGA) [25-27] have been used individually or in blends for the fabrication of barrier membranes. The addition of bioactive ceramics such as hydroxyapatite (HA) [28] and tricalcium phosphate (TCP) [29] has also been proven to improve the mechanical and biological properties of barrier membranes. Composite materials have allowed the fabrication of tailor-made barrier membranes that exhibit positive synergistic effects [30]. The compatibilization strategies for the fabrication of polymer blends and polymer-ceramic composites have traditionally involved the use of solvents and high temperatures [31]. Unfortunately, these techniques have several limitation such as: a) non-homogeneous mixing [32], b) denaturing of biomolecules [33], and c) increased operation costs due to slow reaction kinetics [34]. These drawbacks necessitate the development and use of safe, low-temperature, solid-state techniques for the processing of biocomposites.

This study intends to validate the fabrication approach of novel biodegradable polymer-bioactive ceramic barrier membranes for use in GBR. A low-temperature, solid-state, blending technique called cryomilling was employed to generate polymer-ceramic biocomposites made of PLA, CS, and TCP. As mentioned previously, PLA-based membranes have produced considerable success when used for GBR. CS, apart from being resorbable and biocompatible, was also chosen for its antimicrobial properties [35]. Moreover, the role of CS in cell adhesion, proliferation, and osteoblast differentiation has been well documented [36-38]. TCP was used as a synthetic bone substitute material to induce the regeneration of bone tissue [39-41]. Even though these materials have been previously used in the fabrication of scaffolds for GBR, the potential of a biocomposite with these materials remains unexplored.

In this study, electrospinning was employed to generate barrier membranes due to its previous success in bone tissue engineering (BTE) applications [42-44]. RAW 264.7 murine macrophages were used to evaluate the cytocompatibility of the generated biocomposites and investigated by CellTiter-Blue® (CTB®) cell viability assay. Further, the electrospun membranes were evaluated for their capability to support the growth of MG63 cells using CTB® cell viability assay. A live/dead viability assay was also conducted, and the fibers were qualitatively analyzed using coherent anti-Stokes Raman Spectroscopy (CARS) and fluorescence microscopy. Also, a 21-day *In vitro* degradation studied was performed and analyzed using Raman spectroscopy.

## 4.3. Materials and Methods

### *4.3.1. Fabrication of PLA/CS/TCP Biocomposites*

PLA (Purasorb PL 10; Corbion Purac), CS (448877-50G, Medium $M_w$; Sigma-Aldrich), TCP (C5267-100G, 34.0-40.0% Ca basis; Sigma-Aldrich) were cryomilled to generate

polymer-ceramic powder composites. Compositions of the blends prepared are as follows: A0: 100% PLA; A1: 70% PLA, 30% CS; A2: 68% PLA, 30% CS, 2% TCP; A3: 66% PLA, 30% CS, 4% TCP. Briefly, exact quantities of each material were transferred into a polycarbonate vial with stainless-steel impactors and end plugs. The vial was then loaded into the freezer mill (6870; SPEX, Metuchen, NJ, USA) which was maintained at a temperature of -196 ℃ using liquid nitrogen. Each composition was cryomilled for 20 mins in 4 cycles. A cooling time of 1 min was allowed between successive cycles and a precool time of 4 mins was utilized to ensure homogeneity in temperature at the time of milling. A total sample weight of 5 g was used for every run. The samples were stored in a silica-filled desiccator for at least 48 h at room temperature before further processing.

*4.3.2. Fabrication of PLA/CS/TCP Biocomposite Membranes*

Electrospinning was employed to generate fibrous membranes using the generated biocomposites. Trifluoroacetic acid (TFA) (O4902-100; Fisher Scientific) was used as the common solvent. In a typical process, 18-20 w/v% of the cryomilled powder was added to 5 ml of TFA, and the solution was stirred using a magnetic stirrer at 25 ℃ for at least 24 h to ensure homogeneous dispersion of the bioceramic particles. The solution was then transferred to a 5 ml syringe with an 18G PrecisionGlide needle (Becton-Dickinson, Franklin Lakes, NJ, USA). A stationary copper plate covered with aluminum foil was used as the collector plate. The needle tip to collector distance was set to 13 cms, and the potential difference was adjusted between 15-17.5 kV as needed with a constant solution flow rate of 0.05 ml $min^{-1}$. All the electrospun membranes were kept at 40 ℃ in a vacuum oven for 24 h before the further investigation was performed.

*4.3.3. Scanning Electron Microscopy*

The morphology of the samples was analyzed using a JEOL JCM-6000Plus NeoScope Benchtop scanning electron microscope (SEM) (JEOL, Peabody, MA, USA). Accelerating voltages of 10-15 kV were used depending on the requirement. The fiber diameter was measured using JCM-6000 software version 1.1. The mean diameters of the barrier membranes were calculated using thirty measurements from three independent samples. All diameter values have been reported as mean ± standard deviation (SD).

*4.3.4. X-Ray Diffraction Analysis*

X-Ray Diffraction (XRD) analysis was performed to confirm the occurrence of a homogenous composite blend. The biocomposites were analyzed using the Rigaku Miniflex 600 XRD analysis unit (Tokyo, Japan). The voltage and current applied were 30 kV and 15 mA respectively. A scintillator counter (SC-70) was used as the detector. The scan ranged from 3 to 80 degrees with steps of 0.02 degrees. PDXL (version 2.1.3.4.) was used to analyze the data.

*4.3.5. Differential Scanning Calorimetry*

Differential scanning calorimetry (DSC) (Phoenix, NETZSCH Instruments, Burlington, MA, USA) was used for thermal characterization of the generated biocomposite powders. In a typical measurement process, 8 mg of the sample was analyzed using aluminum pans and argon purge gas. An empty aluminum crucible was used as the reference. In order to avoid the degradation of CS, the method of Sakurai et al. was used with modifications [45]. Suyatma et al. have reported the success of this method for the thermal characterization of PLA/CS biodegradable

films [46]. The samples were quenched to at -10 K $min^{-1}$ to -30 ℃ before being heated to 190 ℃ at the same rate. The samples were held at 190 ℃ for 1 min before being cooled down to -30 ℃. The samples were then held at -30 ℃ for 3 mins before the second heating cycle in which the samples were heated up to 250 ℃. The second heating scan was used to identify the glass transition and melting temperatures of the cryomilled blends. Proteus Thermal Analysis version 6.1.0 was used for the analysis.

### *4.3.6. Powder Preparation for Cell Cytotoxicity Study*

Powders A0, A1, A2 and A3 were weighed on a precision scale and were suspended in a standard Dulbecco's Modified Eagle's Medium (DMEM) at a concentration of 0.84% w/v (0.05 g powder in 6 ml of standard medium), creating respectively StockA0, StockA1, StockA2, and StockA3 medium. Each stock medium was then transferred into a Pyrex bottle and autoclaved at 121 ℃ for 15 mins. Stock media were cooled to room temperature, then each of them was diluted to a final concentration of 3 µl/ml, 10 µl/ml, 30 µl/ml and 50 µl/ml using the standard medium. The new solutions were named CompA0, CompA1, CompA2 and CompA3 medium.

### *4.3.7. Cell Cytotoxicity Study using RAW 264.7 Murine Macrophages*

RAW 264.7 murine macrophage cell line was used to test the cytocompatibility of CompA0, CompA1, CompA2 and CompA3 medium, in different concentration as explained in section 4.3.6. Cytotoxicity was tested by treating macrophages with powders suspensions in standard medium, as performed by Varmette et al. (2008) [47]. The cells, at passage 12, were cultivated in standard DMEM medium (Sigma-Aldrich, Irvine, UK) supplemented with 10% Fetal

Bovine Serum (FBS) (Invitrogen, Paisley, UK) and 1% penicillin/streptomycin (100U/ml/100 µg/ml, Invitrogen, Paisley, UK). When confluent, cells were trypsinized, counted and subsequently seeded into 96-well plate at the concentration of $3x10^4$ cells/well in a volume of 100 µl of the medium. Cells were then incubated for 24 hours at 37 °C, 5% $CO_2$, and subsequently exposed to CompA0, CompA1, CompA2 and CompA3 and added to cell culture at final concentrations of 3 µl/ml, 10 µl/ml, 30 µl/ml and 50 µl/ml in 100 µl of medium/well. Cells were incubated for 24 h at 37 °C, 5% $CO_2$ and at the end of the incubation time, cells were imaged using bright microscopy (BF). Then, CompA0, CompA1, CompA2 and CompA3 media were replaced with fresh, standard medium and cells were incubated for further 24 h and 48 h, before proceeding with the CTB® cell viability assay.

*4.3.8. CellTiter-Blue® Cell Viability Assay on RAW 264.7 Murine Macrophages*

Cell viability was investigated using CTB® cell viability assay (Promega, Southampton, UK) after 24 and 48 h from the end of the treatment with Comp A0, A1, A2 and A3 media. Briefly, 20 µl/well of the reagent was added to cells grown in a 96-wells plate, according to the manufacturer's protocol. Then, cells were incubated in an incubator at 37 °C, 5% $CO_2$ for 3 h. At the end of the incubation time, the supernatant of each well was transferred to a fresh 96 wells black plate, glass bottom, and fluorescence was measured with a microplate reader (Modulus™ II Microplate Multimode Reader, Turner Biosystems, Sunnyvale, California, USA) at 560/690 nm. Cells grown in the standard medium were used as controls and samples were investigated in triplicates. All data are expressed as mean values along with minimun and maximum values. At the end of the incubation time with CompA0, A1, A2 and A3 media, cell morphology was evaluated using BF microscopy (Leica Microsystem, Milton Keynes, UK), at 200x magnification.

*4.3.9. MG63 Cell Seeding on Electrospun Membranes*

MG63 Human Osteosarcoma cell line was cultivated in standard DMEM medium (Sigma-Aldrich, Irvine, UK) supplemented with 10% FBS (Invitrogen, Paisley, UK) and 1% penicillin/streptomycin (100U/ml/100 µg/ml, Invitrogen, Paisley, UK). When confluent, cells were trypsinized, counted, suspended into 30 µl of medium and subsequently seeded into scaffolds at the concentration of $7.5x10^4$ MG63 cells/scaffold. Before cells seeding, scaffolds were placed one per well in a sterile 48 wells plate, UV irradiated for 15 mins and pre-soaked into standard cell medium for 10 mins. At the end of the soaking time, the medium was removed, and cells were seeded. Cell attachment was favored by incubating scaffolds at 37 °C, 5% CO2 for 15 mins and then 500 µl of standard medium were gently added to each scaffold. The day after, scaffolds were gently transferred to a fresh 48 wells plate, to avoid any contribution of cells that have not grown directly on the scaffolds, as done by Tampeiri et al. (2014) [48]. The medium was changed every 2 days. MG63 cells viability and proliferation was investigated after 3 and 7 days. All cell manipulation procedures were conducted in a sterile laminar flow hood.

*4.3.10. Cell Viability Investigation by Fluorescence Microscopy*

Cell viability was qualitatively investigated using LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells (Invitrogen, Paisley, UK), as suggested by the manufacturer's protocol. Briefly, $7.5x10^4$ MG63 cells were seeded into each electrospun scaffold as previously described and then incubated at 37 °C, 5% $CO_2$ for 3 and 7 days respectively. At the end of the incubation period, the medium was removed, and scaffolds were incubated with Calcein acetoxymethyl (Calcein-AM) 2 µM plus Ethidium homodimer-1(EthD-1) 4 µM for 15 mins at 37 °C, 5% CO2, in the dark. At the end of the incubation time, each scaffold was gently washed with 1 ml of PBS

1X. Fluorescent dyes were excited and imaged using two-photon emission fluorescence (TPEF), while scaffolds fibers were depicted using CARS. The laser used is a HighQ picotrain laser unit providing a 1064 nm and a 532 nm beam [49]. The OPO beam was set at 813 nm. The Filters used to read the green fluorescent were Semrock 515/42 combined with a 535/40 band-pass filter. For the Red fluorescent marker, the filters used were 609/54 and 640/14 band-pass filters while for the non-resonant CARS, the 660/13 band-pass filter was used.

*4.3.11. CellTiter-Blue® Cell Viability Assay on MG63*

MG63 cell viability was investigated using CTB® cell viability assay (Promega, Southampton, UK), respectively after 3 and 7 days from cells seeding into scaffolds. Cell viability was determined by using the indicator dye Resazurin, which is reduced into highly fluorescent Resorufin by viable cells, while non-viable cells do not to perform the reaction and so no fluorescence is registered. Briefly, $7.5x10^4$ MG63 cells were suspended into 500 µl of the medium and seeded into each electrospun scaffold as previously described, in a 48 wells plate. Scaffolds were then incubated at 37 °C, 5% $CO_2$ for 3 and 7 days respectively. At the end of the incubation period, 100 µl of CellTiter-Blue solution were added to scaffolds, which were incubated in an incubator at 37 °C, 5% $CO_2$ for 3 h. At the end of the incubation time, the supernatants of each well together with the scaffolds were transferred into a fresh micro vial which was centrifuged at 1,000 rpm for a minute. The supernatants were taken and put into a fresh micro vial and mixed by vortex. Then, 100 µl of the supernatants were transferred into a dark glass bottom plate, and fluorescence was measured with a microplate reader (Modulus™ II Microplate Multimode Reader, Turner Biosystems, Sunnyvale, California, USA) at 560/690 nm. Cells grown in standard medium

were used as controls and samples were investigated in triplicate. All data are expressed as mean values along with minimun and maximum values.

*4.3.12. In vitro Degradation of Electrospun Membranes*

Electrospun membranes of A0, A1, A2, and A3 were cut into 4 different pieces and UV irradiated for 15 mins for sterilization. Then, in order to study material degradation, samples were incubated in distilled, purified, sterile water at 37 °C, 5% $CO_2$ for 7, 14 and 21 days. At each time point, samples were investigated using Raman spectroscopy. The results are the products of 20 different spectra from 20 different locations on each sample.

*4.3.13. Statistical Analysis*

Results of the CTB® cell viability assay were analyzed using PRISM® version 7.0 (GraphPad Software, San Diego, CA, USA) with 95% confidence intervals (CI) of the difference. Shapiro-Wilk W test was used to check the normality of the data because of its higher power for small sample size when compared to the other normality tests [50]. Two-way ANOVA was performed to examine the influence of two different categorical independent variables on a single dependent variable. Post-hoc Tukey Honestly Significant Difference (HSD) test was utilized to perform multiple pairwise comparisons when the two-way ANOVA confirmed statistical significance. All the analyses were conducted with the designated Type I error rate of 0.05 [51].

## 4.4. Results and Discussion

### *4.4.1. Morphology of Cryomilled Composites*

As previously mentioned in the introduction, biocomposites serve as a convenient option to fabricate barrier membranes that could elicit desirable biological responses from the human body. In this study, cryomilling, a low-temperature solid-state technique was used for the generation of polymer-polymer and polymer-ceramic powder blends. Cryomilling has repeatedly been used in the past to develop homogeneous blends of immiscible polymers [52]. It has been shown to produce finer grain structures in relatively shorter processing times [53]. Recently, Lim et al. employed cryomilling for the fabrication of polycaprolactone/TCP composites and reported that homogeneous dispersion could be attained even at high loading percentages of TCP [54]. Apart from eliminating the need for solvents and high temperatures, cryomilling also requires lesser energy to induce a material fracture as the entire process as the milling is done below the glass transition temperature of the polymers. Fig. 11 shows a micrograph of a cryomilled polymer–ceramic blend (A3) that was generated in this study. The composite powder was found to be made up of particles with sharp edges, and the particle size of the composite blend was visibly smaller than the particle size of the materials before cryomilling. The agglomeration observed, is because of the presence of electrostatic, steric and van der Waals forces between the particles [55-57].

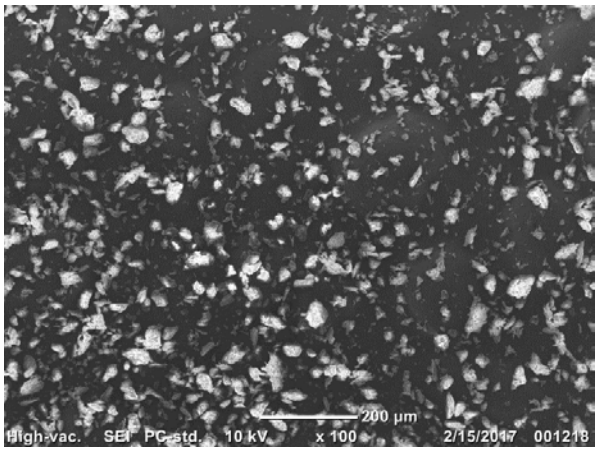



**Fig. 11.** SEM image of PLA/CS/TCP cryomilled composite particle (A3)

*4.4.2. X-Ray Diffraction Analysis of Cryomilled Powders*

XRD was utilized to confirm the occurrence of a homogeneous blend PLA, CS, and TCP. Fig. 12 shows the XRD profiles for as-received CS, and TCP along with the profiles for the composite blends prepared by cryomilling. The first characteristic diffraction peak for PLA (A0) occurs at a 2θ value of 16.38° and the second major peak can be seen at a 2θ value of 18.76°. For pure CS, the only evident characteristic diffraction peak occurs at a 2θ value of 19.48°. TCP has two distinct diffraction peaks close to a 2θ value of 30°. The XRD profiles of A1, A2 and, A3 showcase PLA peaks at 2θ values of 16.38° and 18.76° indicating the presence of PLA. Also, the presence of CS can be confirmed by observing the profiles of the composite materials close to a 2θ value of 19.48°. It can clearly be observed that the profiles of the composite materials show relatively higher intensities at those points indicating the presence of CS. Similarly, the composite blends consisting of TCP particles have profiles with relatively higher intensities at 2θ values close to 30° when compared to the blends without the ceramic component. In conclusion, it can be said that the cryomilling process generated a homogeneous blend of materials as the composite blends have a unique profile that is different from the profile of the individual constituent materials.

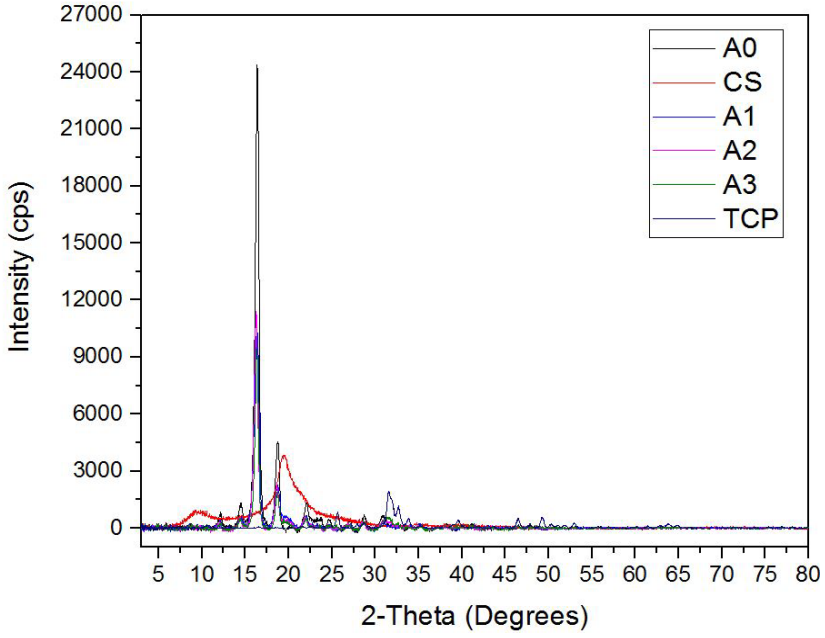



**Fig. 12.** XRD profiles of composite blends along with their individual components

*4.4.3. Differential Scanning Calorimetry Analysis of Cryomilled Composites*

DSC was used for thermal characterization of the biopolymer-bioceramic powder blends that were prepared by cryomilling. The DSC profile of PLA (A0) (Fig. 13) clearly resembles a typical profile of a semi-crystalline polymer. The glass transition occurs at a temperature of 59 °C, and a cold crystallization can be seen as an exothermic peak beginning at around 150 °C. The melting point of pure PLA was identified to be 179.5 °C which was close to the value provided by the manufacturer. It was observed that the addition of CS and TCP increased the glass transition temperature of PLA (Table 2). Based on observations made by Tanase et al. (2014), it was hypothesized that the movement of PLA chains was hindered by the addition of CS and TCP [58]. It was also identified that the melting temperature of the membranes decreased with the addition of TCP particles indicating the development of less crystalline materials. It was reasoned that the

TCP particles acted as obstacles and prevented the growth of crystal structures [59]. Table 1 also shows the melting point data obtained from the second heating scan.

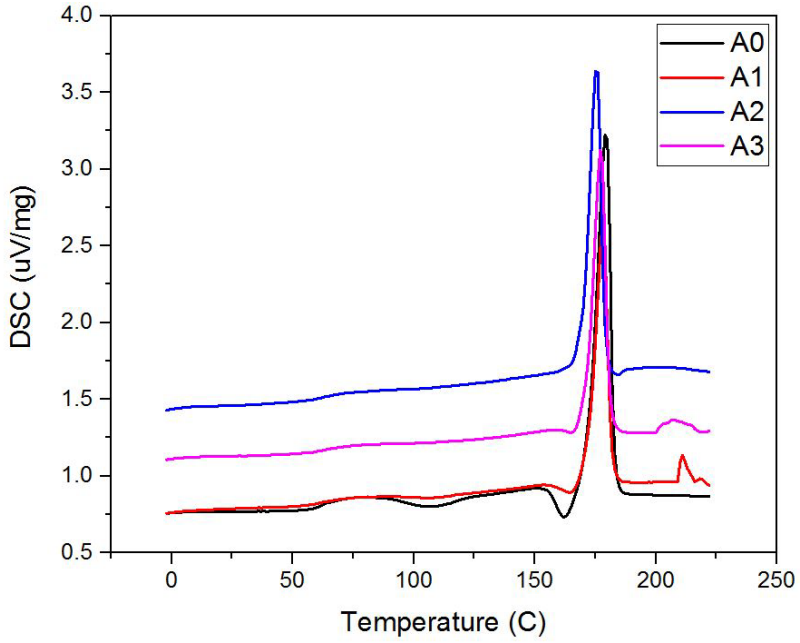



**Fig. 13.** DSC second heating scans of the cryomilled composites

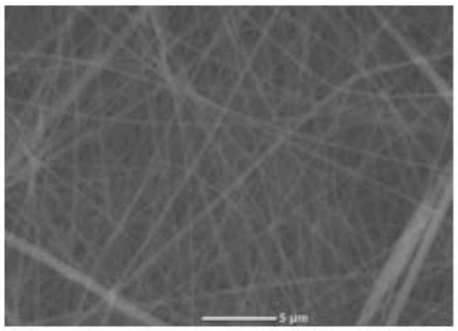
**Table 2.** DSC glass transition and melting data

| Material | Glass Transition (°C) | Melting Point (°C) |
|---|---|---|
| A0 | 59 | 179.5 |
| A1 | 60.1 | 178 |
| A2 | 63.3 | 175.4 |
| A3 | 63.9 | 177.2 |

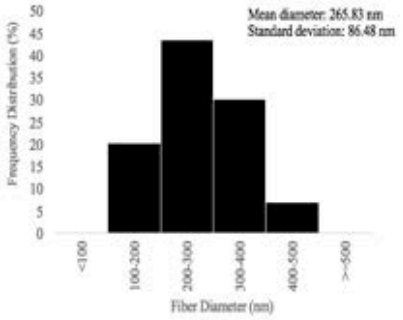
*4.4.4. Morphology of Electrospun Barrier Membranes*

In this study, fibrous membranes were generated using electrospinning. Bhardwaj et al. (2010) describe electrospinning as "a technique that uses electrostatic forces to generate thin fibers from polymer solutions" [60]. In the past, researchers have had tremendous success is fabricating polymeric fibrous scaffolds for tissue engineering applications [61]. The processing conditions in electrospinning, including viscosity, molecular weight of polymer, applied voltage, needle tip-collector plate distance, solution flow rate, have been shown to have a significant effect on the morphology of the fibers generated [62]. The observations from the pilot studies revealed the inability of solvents such as dichloromethane (DCM), dimethylformamide (DMF), and chloroform to act as the common solvent. This phenomenon was attributed to their ionic character in dissolved state and three-dimensional networks of strong hydrogen bonds [63]. It was also observed that CS was insoluble at neutral and alkaline pH but dissolved in acidic media [64].

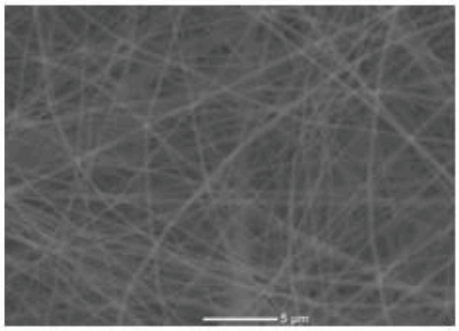
Based on observations made during the pilot study, 18-20 w/v% was identified to be the optimum concentration for electrospinning A0, A1, A2, and A3. Fig. 14 shows SEM images of the fibers that were generated from each blend along with their corresponding diameter distribution profiles. While comparing the fiber diameters of pure A0 and A1, it was obvious that the introduction of CS resulted in the generation of relatively thicker fibers. This was found to be in agreement the observations made by Xu at al. (2009) [65]. Hence, it was concluded that the increase in PLA content resulted in the disappearance of beads and resulted in a finer fiber morphology. It was also noted that as the content of TCP particles increased in the composite matrix, the fiber diameter shifted to a higher range indicating that the TCP particles were responsible for this increase. Yang et al. (2008) have reported similar observations while electrospinning PVA/CS with HA fillers [66].



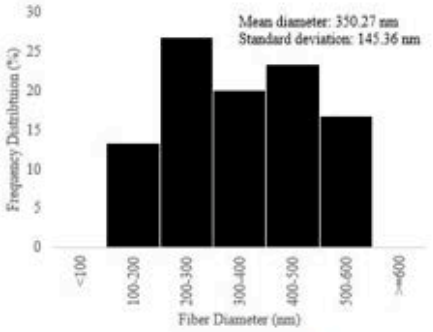

Micrographs of electrospun membrane made of A0



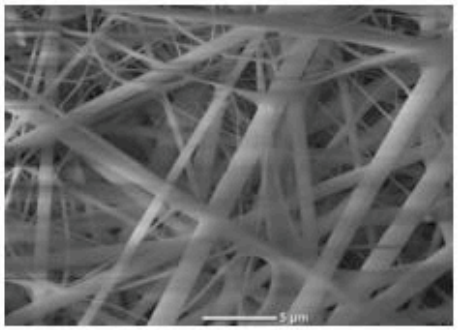

Diameter distribution of A0 fibers



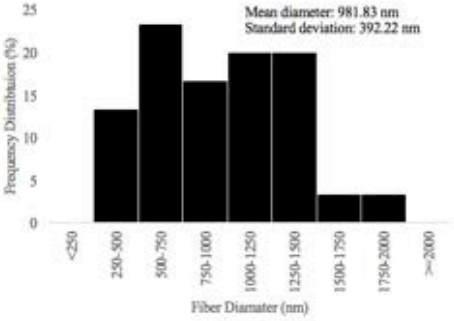

Micrographs of electrospun membrane made of A1



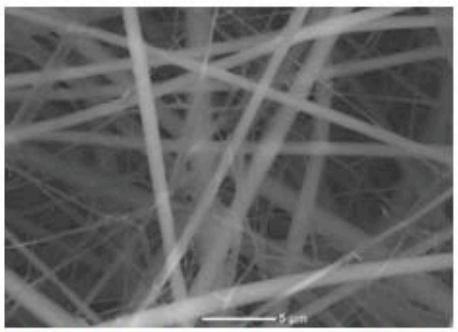

Diameter distribution of A1 fibers



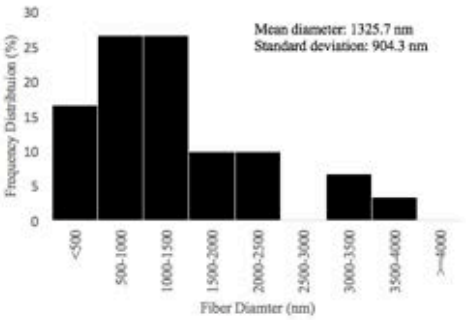

Micrographs of electrospun membrane made of A2




Diameter distribution of A2 fibers




Micrographs of electrospun membrane made of A3




Diameter distribution of A3 fibers

**Fig. 14.** SEM images and diameter distributions of electrospun barrier membranes

*4.4.5. Cell Viability Assay on RAW 264.7 Murine Macrophages*

The cytotoxicity of the composite cryomilled powders blend A0, A1, A2 and A3 was tested on murine macrophage RAW 264.7 cell line, as described in the Materials and Methods section. Cell viability was determined by CTB® assay (Fig. 15 (a, b)). Results indicate that 24 h after returning in standard medium cultivation, cell viability is statistically reduced respect to the control for all the concentrations tested. However, 48 h after returning in standard medium cultivation, the gap in cell viability between cells cultured with different cryomilled powders and controls is reduced and no statistical differences was notice between controls and cells cultured with powders. After 24 h of incubation with composite cryomilled powders blend A0, A1, A2 and A3, the cell morphology was investigated using BF microscopy (Fig. 16 (a – e)). The investigation revealed that powders particles, when in the medium, tended to agglomerate creating bigger structures, and macrophages grew around or all over them. There were no obvious differences in the morphology between macrophages cultured with composite cryomilled powders and the controls.

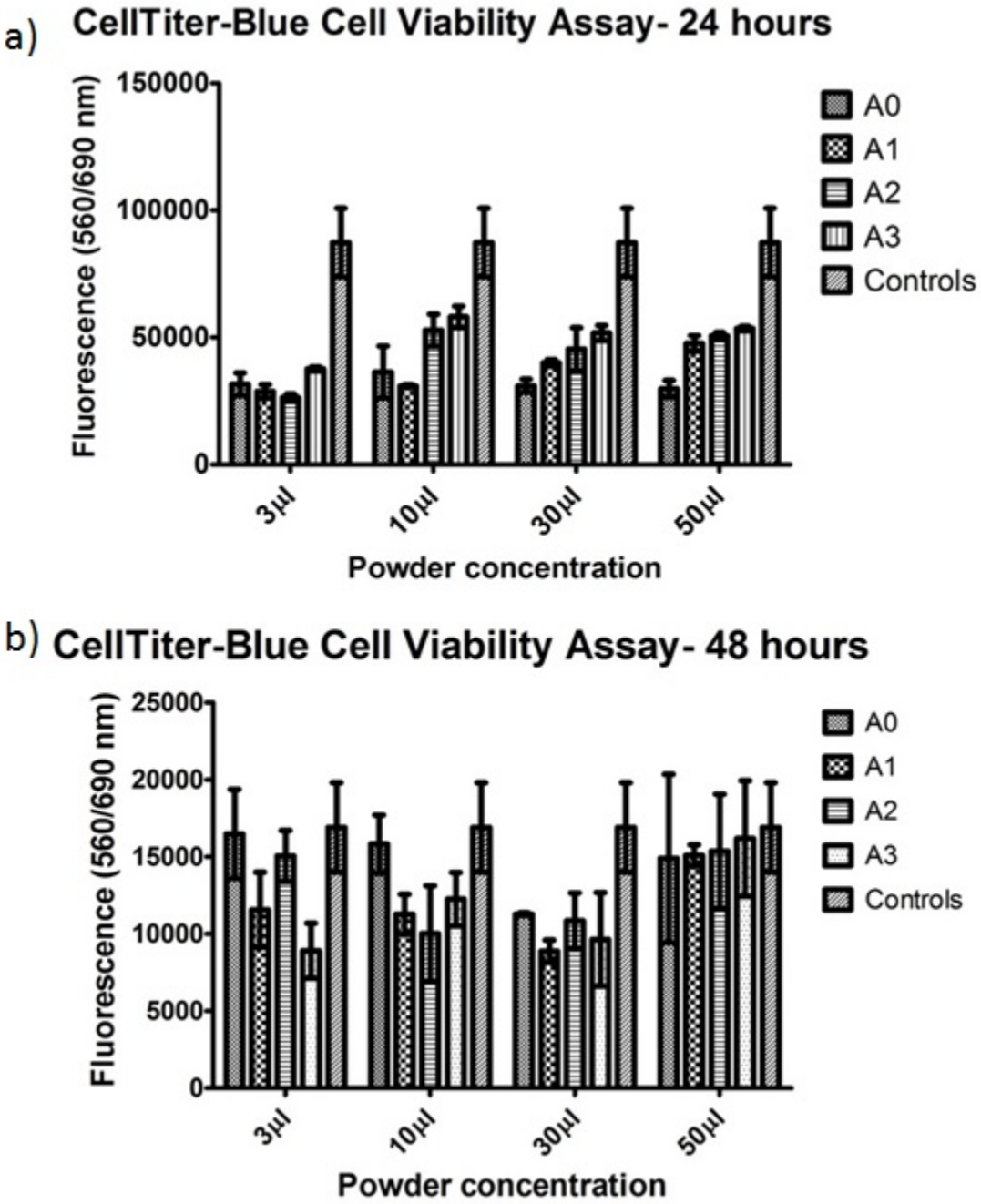
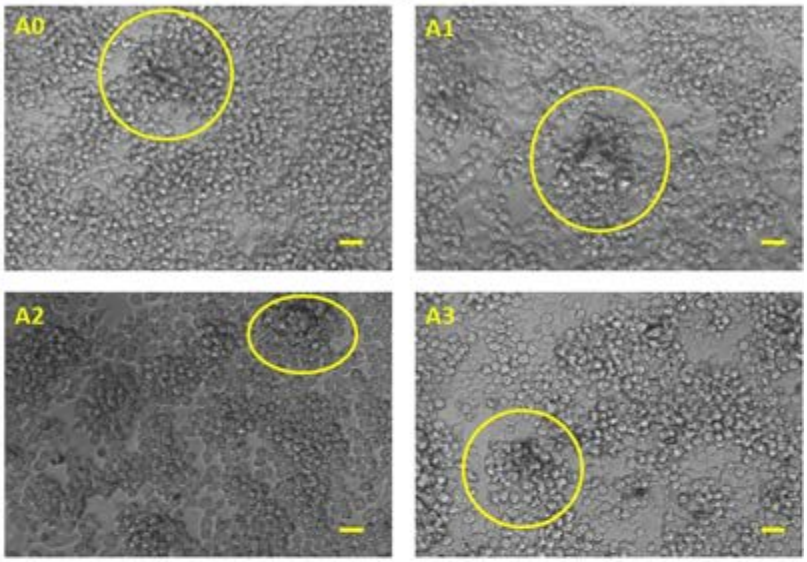
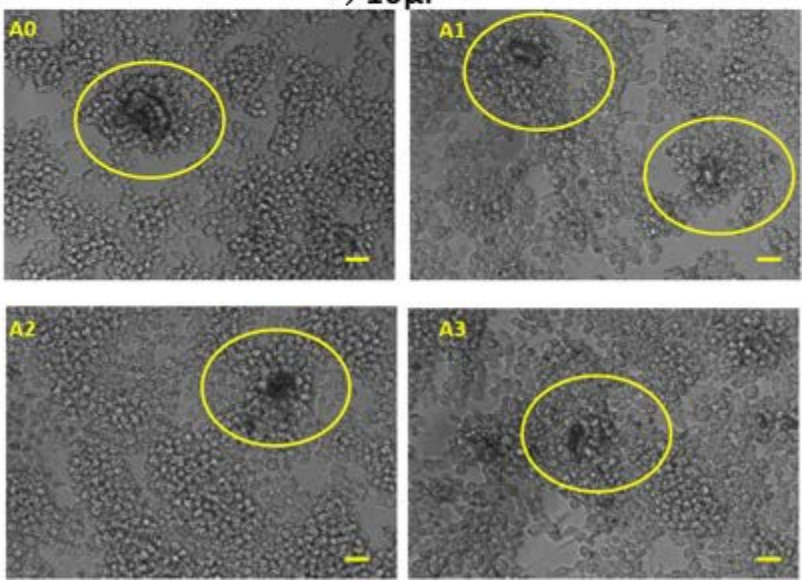



**Fig. 15.** CTB® assay performed on RAW 264.7 cell line incubated with composite cryomilled powders blend A0, A1, A2 and A3, at different concentrations, after 24 h (a) and 48 h (b) from returning in standard medium conditions. Results are indicated as mean with min. and max. values.

a) 3µl




b) 10µl



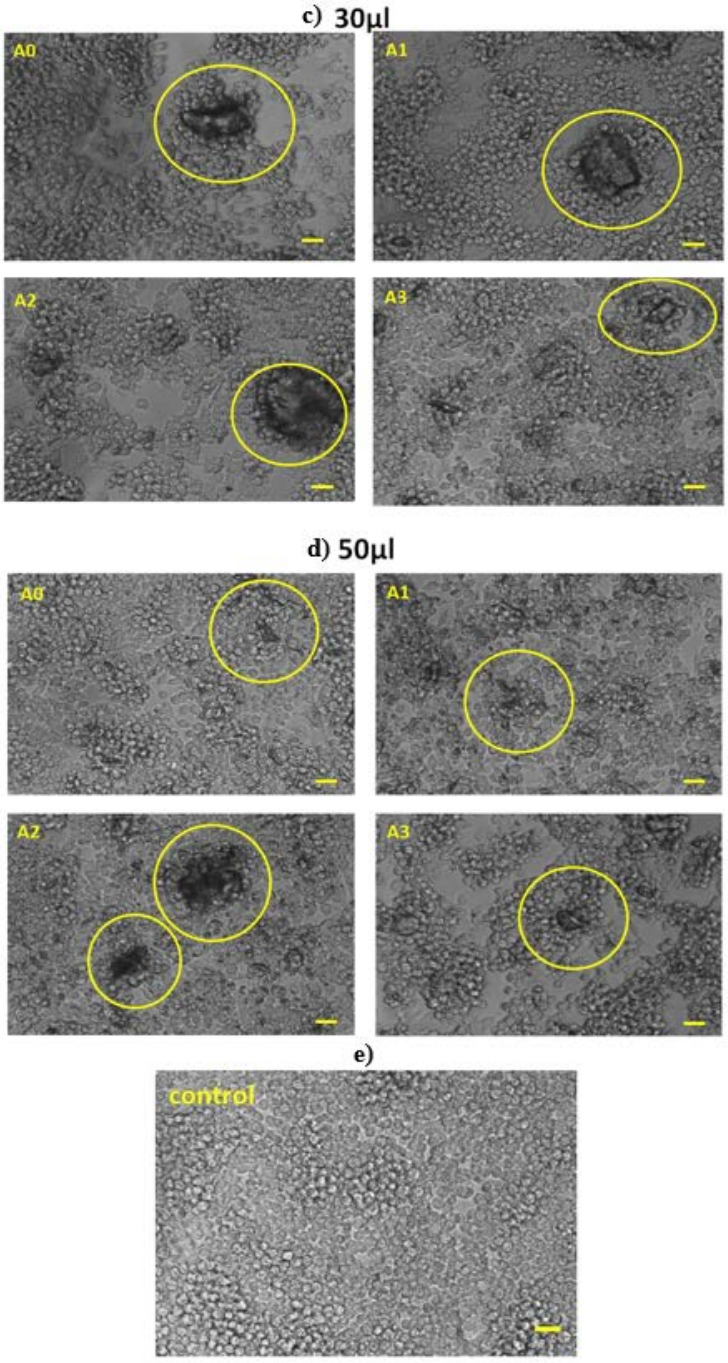



**Fig. 16.** BF microscopy of RAW 264.7 macrophages incubated for 24 hours with different composite cryomilled powders at concentrations a) 3 µl/ml, b) 10 µl/ml,
c) 30 µl/ml, d) 50 µl/ml, e) control; Magnification = 200X; Scale bar = 20µm.

*4.4.6. MG63 Cell Viability Investigation by Fluorescence Microscopy*

The viability of MG63 cells was qualitatively examined at day 3 and 7 from cell seeding into scaffolds by fluorescence in green, red and CARS microscopy. All the scaffolds show high cell viability and low number of dead cells, as shown in Fig. 17, where living cells are stained in green and dead cells are stained in red. Cells were able to colonize the scaffolds, growing and dividing on them. Moreover, scaffolds A2, A3 and A3 appeared more suitable for cell attaching than scaffold A0, as indicated by the pictures which show a higher number of cells growing inside the scaffold fibers. Scaffolds A1 and A3, in particular, were noticed to have a good cell attachment along scaffold fibers.

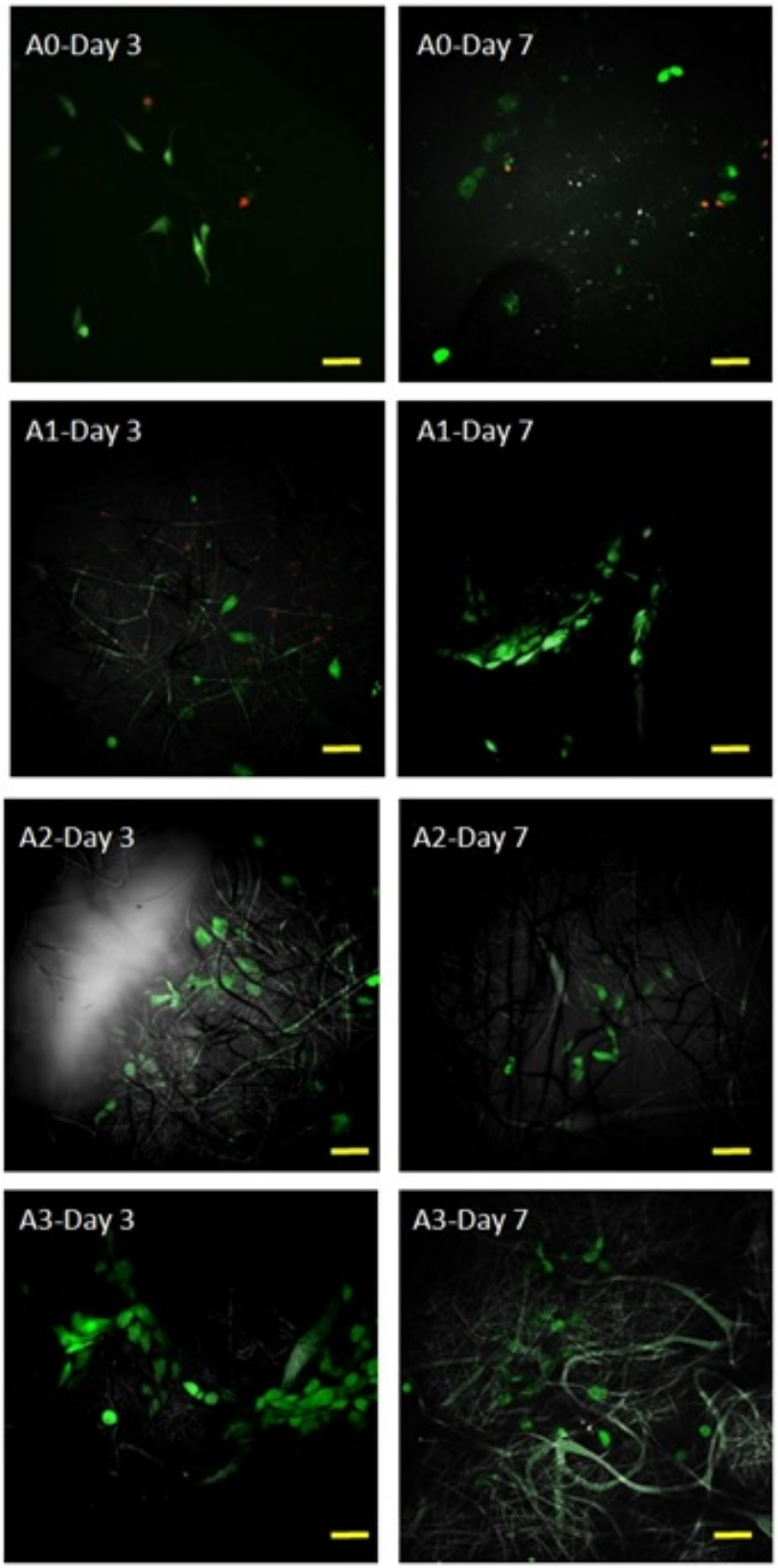



**Fig. 17.** Fluorescence microscopy on MG63 cells seeded into electrospun scaffolds.
Living cells are green, dead cells are red. Scaffolds fibers are depicted using CARS. Scale bar = 25 µm.

*4.4.7. MG63 Cell Viability Investigation by CellTiter-Blue® Assay*

MG63 cell viability was evaluated also by CTB® assay, at day 3 and 7 from cell seeding into scaffolds (Fig. 18). The results confirm the observations from the fluorescence investigation, showing that cells are viable at both the time points investigated. Moreover, there is an increase of registered fluorescence intensity, corresponding to an increase of cell viability, at day 7 respect to day 3, showing that cells are able to integrate inside the scaffolds and to grow on them with the passing of days. No statistical difference in cell viability was noticed between scaffolds types at both the days investigated, indicating that all the scaffolds have a comparable performance.

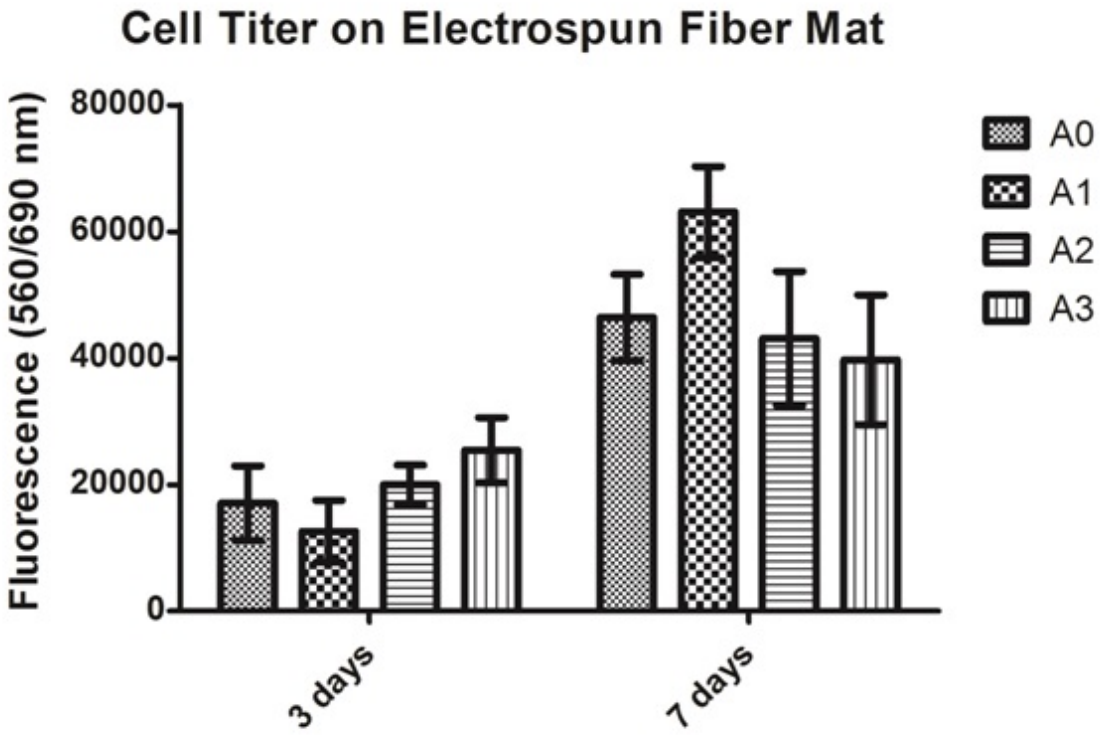



**Fig. 18.** CTB® assay performed on MG63 cells seeded into electrospun scaffolds, after 3 and 7 days from cell seeding. Results are indicated as mean with min. and max. values.

*4.4.8. In vitro degradation study on electrospun membranes*

Fig. 19, 20, 21 show the Raman spectra for PLA, CS and TCP respectively. The spectra obtained from electrospun membranes were used to explain the degradation behavior of the biocomposites. The investigation revealed that A0, made of 100% PLA, showed a poor degradation through time (Fig. 22). The slow degradation of PLA was attributed to the presence of hydrophobic methyl groups [67]. Sample A1 shows a degradation at day 7 which is increased at day 14 and day 21, particularly in the region of 1400-1500 $cm^{-1}$, where the expected degradation is shown (Fig. 23). Sample A2 shows an unusual degradation pattern that suggests that sample is more degraded at day 7 and 14 than at day 21. This could be due to sample flotation as seen in A0 (Fig. 24). Sample A3 shows the expected degradation pattern, and it is more degraded at day 14 and 21 than at day 7 (Fig. 25). It was concluded that the addition of CS favored the degradation of PLA by increasing the hydrophilicity of the membrane [68]. The accelerated degradation can also be explained by the fact that CS is a molecule that dissolves and degrades in acidic environments [69]. The accelerated degradation of A1 and A2 membrane showed confirmed the hypothesis that the TCP particles had reduced the crystallinity of pure PLA membranes.

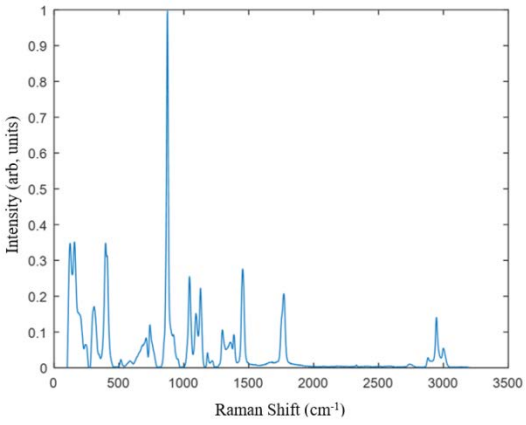



**Fig. 19.** Raman spectra of pure PLA

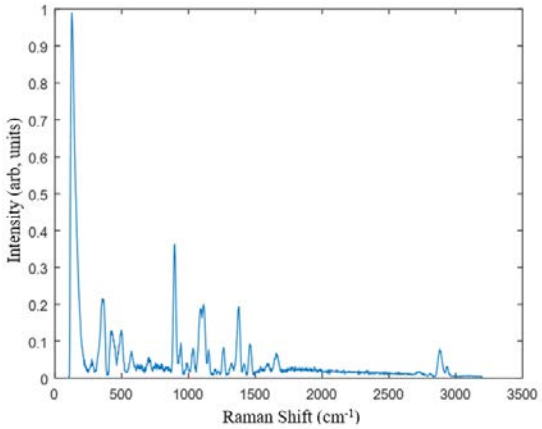



**Fig. 20**. Raman spectra for pure CS

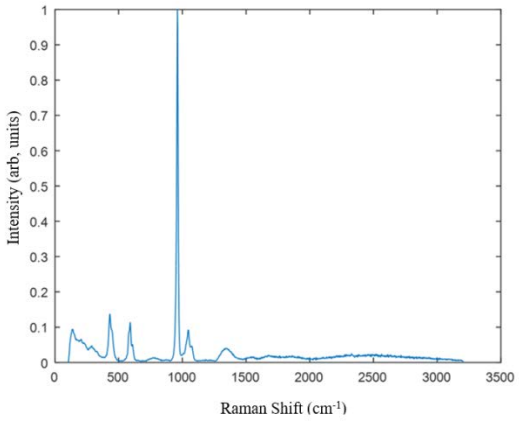



**Fig. 21.** Raman spectra of pure TCP

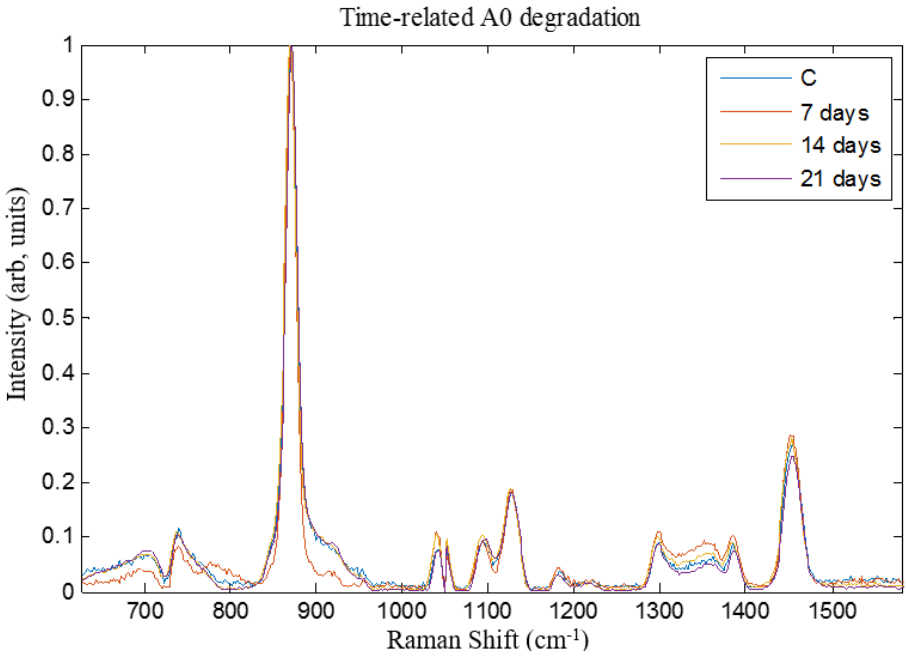



**Fig. 22.** Raman spectra of A0 electrospun membrane

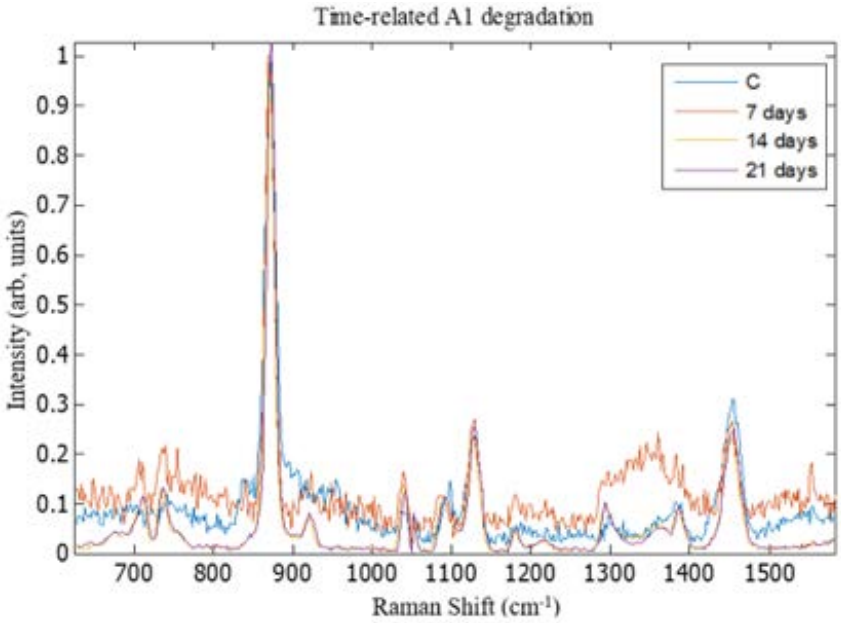



**Fig. 23.** Raman spectra of A1 electrospun membrane

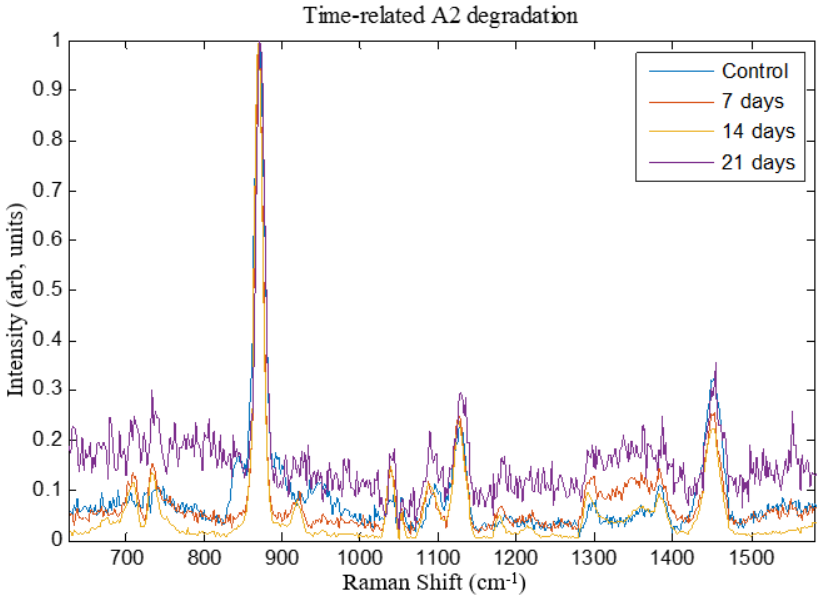



**Fig. 24.** Raman spectra of A2 electrospun membrane

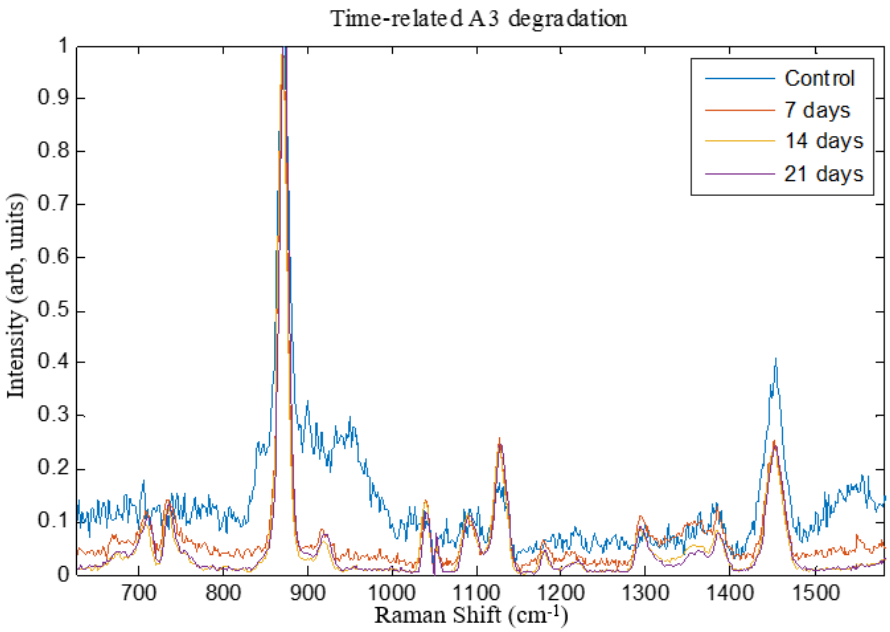



**Fig. 25.** Raman spectra of A3 electrospun membrane

## 4.5. Conclusions

Cytotoxicity test on powders used for scaffolds creation revealed a toxicity for macrophages, as their viability is reduced 24 h after returning in standard conditions after incubation with powders respect to the untreated controls. However, this effect could be due to the tendency of the powders to form large agglomerates when in suspension and not because of the toxicity of the materials used, as all the materials that have been utilized have known biocompatible properties [19-21] [35-38] [39-41]. When nanoparticles aggregate, cytotoxicity could arise from many factors such as particle charge, extent of aggregation and cell type used and it is difficult to define in a generic way which of them are predominant on macrophages cytotoxicity [70, 71]. However, after 48 h from returning in standard medium cultivation, viability of macrophages incubated with powders is comparable with the one of controls. This could be interpreted as a transient toxicity of powders, meaning that when powders are removed from cell medium, cells

recover their viability and proliferation activity. In addition to that, electrospun fibers scaffolds formed by these powders show a good biocompatibility and they appear suitable for osteoblast-like cells adhesion and proliferation. MG63 cells show a good attachment to scaffolds fibers and cells growth increases with the passing of days. Also, the biocomposite membranes showed a better degradation behavior when compared to pure PLA membranes.

### 4.6. References

[1] Eke, P. I., Dye, B. A., Wei, L., Thornton-Evans, G. O., & Genco, R. J. (2012). Prevalence of periodontitis in adults in the United States: 2009 and 2010. *Journal of dental research, 91*(10), 914-920.

[2] Bascones-Martínez, A., Muñoz-Corcuera, M., Noronha, S., Mota, P., Bascones-Ilundain, C., & Campo-Trapero, J. (2009). Host defence mechanisms against bacterial aggression in periodontal disease: Basic mechanisms. *Med Oral Patol Oral Cir Bucal, 14*(12), e680-5.

[3] Zhang, L., Henson, B. S., Camargo, P. M., & Wong, D. T. (2009). The clinical value of salivary biomarkers for periodontal disease. *Periodontology 2000, 51*(1), 25-37.

[4] Moses, O., Pitaru, S., Artzi, Z., & Nemcovsky, C. E. (2005). Healing of dehiscence-type defects in implants placed together with different barrier membranes: a comparative clinical study. *Clinical oral implants research, 16*(2), 210-219.

[5] Lee, J. Y., Lee, J., & Kim, Y. K. (2013). Comparative analysis of guided bone regeneration using autogenous tooth bone graft material with and without resorbable membrane. *Journal of Dental Sciences, 8*(3), 281-286.

[6] Beitlitum, I., Artzi, Z., & Nemcovsky, C. E. (2010). Clinical evaluation of particulate allogeneic with and without autogenous bone grafts and resorbable collagen membranes for bone augmentation of atrophic alveolar ridges. *Clinical oral implants research, 21*(11), 1242-1250.

[7] Retzepi, M., & Donos, N. (2010). Guided bone regeneration: biological principle and therapeutic applications. *Clinical oral implants research, 21*(6), 567-576.

[8] Scantlebury, T. V. (1993). 1982-1992: A Decade of Technology Development for Guided Tissue Regeneration*. *Journal of periodontology, 64*(11s), 1129-1137.

[9] Piattelli, A., Scarano, A., Russo, P., & Matarasso, S. (1996). Evaluation of guided bone regeneration in rabbit tibia using bioresorbable and non-resorbable membranes. *Biomaterials, 17*(8), 791-796.

[10] Sculean, A., Nikolidakis, D., & Schwarz, F. (2008). Regeneration of periodontal tissues: combinations of barrier membranes and grafting materials–biological foundation and preclinical evidence: a systematic review. *Journal of Clinical Periodontology, 35*(s8), 106-116.

[11] Mellonig, J. T., & Nevins, M. (1995). Guided bone regeneration of bone defects associated with implants: an evidence-based outcome assessment. *International Journal of Periodontics & Restorative Dentistry, 15*(2).

[12] Aaboe, M., Pinholt, E. M., & Hjorting-Hansen, E. (1995). Healing of experimentally created defects: a review. *British Journal of Oral and Maxillofacial Surgery, 33*(5), 312-318.

[13] Gielkens, P. F., Schortinghuis, J., De Jong, J. R., Raghoebar, G. M., Stegenga, B., & Bos, R. R. (2008). Vivosorb®, Bio-Gide®, and Gore-Tex® as barrier membranes in rat mandibular defects: an evaluation by microradiography and micro-CT. *Clinical oral implants research, 19*(5), 516-521.

[14] Jung, R. E., Fenner, N., Hämmerle, C. H., & Zitzmann, N. U. (2013). Long-term outcome of implants placed with guided bone regeneration (GBR) using resorbable and non-resorbable membranes after 12–14 years. *Clinical Oral Implants Research, 24*(10), 1065-1073.

[15] Kuo, S. M., Chang, S. J., Chen, T. W., & Kuan, T. C. (2006). Guided tissue regeneration for using a chitosan membrane: an experimental study in rats. *Journal of Biomedical Materials Research Part A, 76*(2), 408-415.

[16] Lee, E. J., Shin, D. S., Kim, H. E., Kim, H. W., Koh, Y. H., & Jang, J. H. (2009). Membrane of hybrid chitosan–silica xerogel for guided bone regeneration. *Biomaterials, 30*(5), 743-750.

[17] Shin, S. Y., Park, H. N., Kim, K. H., Lee, M. H., Choi, Y. S., Park, Y. J., ... & Lee, S. J. (2005). Biological evaluation of chitosan nanofiber membrane for guided bone regeneration. *Journal of periodontology, 76*(10), 1778-1784.

[18] Sheikh, Z., Qureshi, J., Alshahrani, A. M., Nassar, H., Ikeda, Y., Glogauer, M., & Ganss, B. (2017). Collagen based barrier membranes for periodontal guided bone regeneration applications. *Odontology, 105*(1), 1-12.

[19] Miller, N., Penaud, J., Foliguet, B., Membre, H., Ambrosini, P., & Plombas, M. (1996). Resorption rates of 2 commercially available bioresorbable membranes. *Journal of clinical periodontology, 23*(12), 1051-1059.

[20] Araujo, M. G., Berglundh, T., & Lindhe, J. (1998). GTR treatment of degree III furcation defects with 2 different resorbable barriers An experimental study in dogs. *Journal of clinical periodontology, 25*(3), 253-259.

[21] Hutmacher, D., Hürzeler, M. B., & Schliephake, H. (1996). A review of material properties of biodegradable and bioresorbable polymers and devices for GTR and GBR applications. *International Journal of Oral & Maxillofacial Implants*, *11*(5).

[22] Santis, R., Gloria, A., Russo, T., D'Amora, U., D'Antò, V., Bollino, F., & Ambrosio, L. (2013). Advanced composites for hard-tissue engineering based on PCL/organic–inorganic hybrid fillers: From the design of 2D substrates to 3D rapid prototyped scaffolds. *Polymer Composites*, *34*(9), 1413-1417.

[23] Domingos, M., Intranuovo, F., Russo, T., De Santis, R., Gloria, A., Ambrosio, L., & Bartolo, P. (2013). The first systematic analysis of 3D rapid prototyped poly (ε-caprolactone) scaffolds manufactured through BioCell printing: the effect of pore size and geometry on compressive mechanical behaviour and in vitro hMSC viability. *Biofabrication*, *5*(4), 045004.

[24] De Santis, R., Russo, A., Gloria, A., D'Amora, U., Russo, T., Panseri, S., & Wilde, C. J. (2015). Towards the design of 3D fiber-deposited poly (caprolactone)/iron-doped hydroxyapatite nanocomposite magnetic scaffolds for bone regeneration. Journal of biomedical nanotechnology, 11(7), 1236-1246.

[25] Hua, N., Ti, V. L., & Xu, Y. (2014). Biodegradable effect of PLGA membrane in alveolar bone regeneration on beagle dog. *Cell biochemistry and biophysics*, *70*(2), 1051-1055.

[26] Oh, S. H., Kim, J. H., Kim, J. M., & Lee, J. H. (2006). Asymmetrically porous PLGA/Pluronic F127 membrane for effective guided bone regeneration. *Journal of Biomaterials Science, Polymer Edition*, *17*(12), 1375-1387.

[27] Won, J. Y., Park, C. Y., Bae, J. H., Ahn, G., Kim, C., Lim, D. H., ... & Huh, J. B. (2016). Evaluation of 3D printed PCL/PLGA/β-TCP versus collagen membranes for guided bone regeneration in a beagle implant model. *Biomedical Materials*, *11*(5), 055013.

[28] Li, J., Zuo, Y., Cheng, X., Yang, W., Wang, H., & Li, Y. (2009). Preparation and characterization of nano-hydroxyapatite/polyamide 66 composite GBR membrane with asymmetric porous structure. *Journal of Materials Science: Materials in Medicine*, *20*(5), 1031-1038.

[29] Kikuchi, M., Koyama, Y., Takakuda, K., Miyairi, H., Shirahama, N., & Tanaka, J. (2002). In vitro change in mechanical strength of β-tricalcium phosphate/copolymerized poly-L-lactide composites and their application for guided bone regeneration. *Journal of biomedical materials research*, *62*(2), 265-272.

[30] Wang, J., Wang, L., Zhou, Z., Lai, H., Xu, P., Liao, L., & Wei, J. (2016). Biodegradable polymer membranes applied in guided bone/tissue regeneration: A review. *Polymers*, *8*(4), 115.

[31] Koning, C., Van Duin, M., Pagnoulle, C., & Jerome, R. (1998). Strategies for compatibilization of polymer blends. *Progress in Polymer Science*, *23*(4), 707-757.

[32] Allaf, R. M., Rivero, I. V., Abidi, N., & Ivanov, I. N. (2013). Porous poly (ε-caprolactone) scaffolds for load-bearing tissue regeneration: Solventless fabrication and characterization. *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, *101*(6), 1050-1060.

[33] Allaf, R. M., & Rivero, I. V. (2011). Fabrication and characterization of interconnected porous biodegradable poly (ε-caprolactone) load bearing scaffolds. *Journal of Materials Science: Materials in Medicine*, *22*(8), 1843-1853.

[34] Ide, F., & Hasegawa, A. (1974). Studies on polymer blend of nylon 6 and polypropylene or nylon 6 and polystyrene using the reaction of polymer. *Journal of applied polymer science*, *18*(4), 963-974.

[35] Panos I, Acosta N, Heras A. New drug delivery systems based on chitosan. *Curr Drug Discov Technol* 2008; **5**: 333–341.

[36] Seol, Y. J., Lee, J. Y., Park, Y. J., Lee, Y. M., Rhyu, I. C., Lee, S. J., ... & Chung, C. P. (2004). Chitosan sponges as tissue engineering scaffolds for bone formation. *Biotechnology letters*, *26*(13), 1037-1041.

[37] Şenel, S., & McClure, S. J. (2004). Potential applications of chitosan in veterinary medicine. *Advanced drug delivery reviews*, *56*(10), 1467-1480.

[38] Aranaz, I., Mengíbar, M., Harris, R., Paños, I., Miralles, B., Acosta, N., ... & Heras, Á. (2009). Functional characterization of chitin and chitosan. *Current Chemical Biology*, *3*(2), 203-230.

[39] Dvir, T., Timko, B. P., Kohane, D. S., & Langer, R. (2011). Nanotechnological strategies for engineering complex tissues. *Nature nanotechnology*, *6*(1), 13-22.

[40] Fujita, N., Matsushita, T., Ishida, K., Sasaki, K., Kubo, S., Matsumoto, T., ... & Kuroda, R. (2012). An analysis of bone regeneration at a segmental bone defect by controlled release of bone morphogenetic protein 2 from a biodegradable sponge composed of gelatin and β-tricalcium phosphate. *Journal of tissue engineering and regenerative medicine*, *6*(4), 291-298.

[41] Chen, J. P., Tsai, M. J., & Liao, H. T. (2013). Incorporation of biphasic calcium phosphate microparticles in injectable thermoresponsive hydrogel modulates bone cell proliferation and differentiation. *Colloids and Surfaces B: Biointerfaces*, *110*, 120-129.

[42] Sui, G., Yang, X., Mei, F., Hu, X., Chen, G., Deng, X., & Ryu, S. (2007). Poly-L-lactic acid/hydroxyapatite hybrid membrane for bone tissue regeneration. *Journal of Biomedical Materials Research Part A*, *82*(2), 445-454.

[43] Kim, H. W., Lee, H. H., & Knowles, J. C. (2006). Electrospinning biomedical nanocomposite fibers of hydroxyapatite/poly (lactic acid) for bone regeneration. *Journal of biomedical materials research part A*, *79*(3), 643-649.

[44] Mei, F., Zhong, J., Yang, X., Ouyang, X., Zhang, S., Hu, X., & Deng, X. (2007). Improved biological characteristics of poly (L-lactic acid) electrospun membrane by incorporation of Multiwalled carbon nanotubes/hydroxyapatite nanoparticles. *Biomacromolecules*, *8*(12), 3729-3735.

[45] Sakurai, K., Maegawa, T., & Takahashi, T. (2000). Glass transition temperature of chitosan and miscibility of chitosan/poly (N-vinyl pyrrolidone) blends. *Polymer*, *41*(19), 7051-7056.

[46] Suyatma, N. E., Copinet, A., Tighzert, L., & Coma, V. (2004). Mechanical and barrier properties of biodegradable films made from chitosan and poly (lactic acid) blends. *Journal of Polymers and the Environment*, *12*(1), 1-6.

[47] Varmette, E. A., Nowalk, J. R., Flick, L. M., & Hall, M. M. (2009). Abrogation of the inflammatory response in LPS-stimulated RAW 264.7 murine macrophages by Zn-and Cu-doped bioactive sol–gel glasses. *Journal of Biomedical Materials Research Part A*, *90*(2), 317-325.

[48] Tampieri, A., Iafisco, M., Sandri, M., Panseri, S., Cunha, C., Sprio, S., ... & Herrmannsdörfer, T. (2014). Magnetic bioinspired hybrid nanostructured collagen–hydroxyapatite scaffolds supporting cell proliferation and tuning regenerative process. *ACS applied materials & interfaces*, *6*(18), 15697-15707.

[49] Downes, A., Mouras, R., & Elfick, A. (2009). A versatile CARS microscope for biological imaging. *Journal of Raman Spectroscopy*, *40*(7), 757-762.

[50] Razali, N. M., & Wah, Y. B. (2011). Power comparisons of shapiro-wilk, kolmogorov-smirnov, lilliefors and anderson-darling tests. *Journal of statistical modeling and analytics*, *2*(1), 21-33.

[51] Mapstone, B. D. (1995). Scalable decision rules for environmental impact studies: effect size, Type I, and Type II errors. *Ecological applications*, *5*(2), 401-410.

[52] Jonnalagadda, J. B., & Rivero, I. V. (2014). Effect of cryomilling times on the resultant properties of porous biodegradable poly (e-caprolactone)/poly (glycolic acid) scaffolds for articular cartilage tissue engineering. *Journal of the mechanical behavior of biomedical materials*, *40*, 33-41.

[53] Suryanarayana, C. (2001). Mechanical alloying and milling. *Progress in materials science*, *46*(1), 1-184.

[54] Lim, J., Chong, M. S. K., Chan, J. K. Y., & Teoh, S. H. (2014). Polymer Powder Processing of Cryomilled Polycaprolactone for Solvent-Free Generation of Homogeneous Bioactive Tissue Engineering Scaffolds. *Small*, *10*(12), 2495-2502.

[55] Jiang, J., Oberdörster, G., & Biswas, P. (2009). Characterization of size, surface charge, and agglomeration state of nanoparticle dispersions for toxicological studies. *Journal of Nanoparticle Research*, *11*(1), 77-89.

[56] Zhu, Y. G., Li, Z. Q., Zhang, D., & Tanimoto, T. (2006). Abs/iron nanocomposites prepared by cryomilling. *Journal of applied polymer science*, *99*(2), 501-505.

[57] Ye, J., Han, B. Q., & Schoenung, J. M. (2006). Mechanical behaviour of an Al–matrix composite reinforced with nanocrystalline Al-coated B4C particulates. *Philosophical magazine letters*, *86*(11), 721-732.

[58] Tanase, C. E., & Spiridon, I. (2014). PLA/chitosan/keratin composites for biomedical applications. *Materials Science and Engineering: C*, *40*, 242-247. Tanase, C. E., & Spiridon, I. (2014). PLA/chitosan/keratin composites for biomedical applications. *Materials Science and Engineering: C*, *40*, 242-247.

[59] Siqueira, L., Passador, F. R., Costa, M. M., Lobo, A. O., & Sousa, E. (2015). Influence of the addition of β-TCP on the morphology, thermal properties and cell viability of poly (lactic acid) fibers obtained by electrospinning. Materials Science and Engineering: C, 52, 135-143.

[60] Bhardwaj, N., & Kundu, S. C. (2010). Electrospinning: a fascinating fiber fabrication technique. *Biotechnology advances*, *28*(3), 325-347.

[61] Schneider, O. D., Loher, S., Brunner, T. J., Uebersax, L., Simonet, M., Grass, R. N., & Stark, W. J. (2008). Cotton wool-like nanocomposite biomaterials prepared by electrospinning: In vitro bioactivity and osteogenic differentiation of human mesenchymal stem cells. *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, *84*(2), 350-362.

[62] Tan, S. H., Inai, R., Kotaki, M., & Ramakrishna, S. (2005). Systematic parameter study for ultra-fine fiber fabrication via electrospinning process. *Polymer*, *46*(16), 6128-6134.

[63] Geng, X., Kwon, O. H., & Jang, J. (2005). Electrospinning of chitosan dissolved in concentrated acetic acid solution. *Biomaterials*, *26*(27), 5427-5432.

[64] Wu, Q. X., Lin, D. Q., & Yao, S. J. (2014). Design of chitosan and its water-soluble derivatives-based drug carriers with polyelectrolyte complexes. *Marine drugs*, *12*(12), 6236-6253.

[65] Xu, J., Zhang, J., Gao, W., Liang, H., Wang, H., & Li, J. (2009). Preparation of chitosan/PLA blend micro/nanofibers by electrospinning. *Materials Letters*, *63*(8), 658-66.

[66] Jin, Y., Nie, J., Zhou, Y., & Yang, D. (2008). Preparation of Core-Shell Structured CS/PVA-PPC Fibers by Coaxial Electrospinning [J]. *Acta Polymerica Sinica*, *5*, 002.

[67] Gilding, D. K., & Reed, A. M. (1979). Biodegradable polymers for use in surgery - polyglycolic/poly (actic acid) homo-and copolymers: 1. Polymer, 20(12), 1459-1464.

[68] Furuike, T., Nagahama, H., Chaochai, T., & Tamura, H. (2015). Preparation and Characterization of Chitosan-Coated Poly (L-Lactic Acid) Fibers and Their Braided Rope. Fibers, 3(4), 380-393.

[69] Sadigh-Eteghad, S., Talebi, M., Farhoudi, M., Mahmoudi, J., & Reyhani, B. (2013). Effects of Levodopa loaded chitosan nanoparticles on cell viability and caspase-3 expression in PC12 neural like cells. Neurosciences, 18(3), 281-3.

[70] Suresh, A. K., Pelletier, D. A., Wang, W., Morrell-Falvey, J. L., Gu, B., & Doktycz, M. J. (2012). Cytotoxicity induced by engineered silver nanocrystallites is dependent on surface coatings and cell types. Langmuir, 28(5), 2727-2735.

[71] Müller, K. H., Motskin, M., Philpott, A. J., Routh, A. F., Shanahan, C. M., Duer, M. J., & Skepper, J. N. (2014). The effect of particle agglomeration on the formation of a surface-connected compartment induced by hydroxyapatite nanoparticles in human monocyte-derived macrophages. Biomaterials, 35(3), 1074-1088.

# CHAPTER V

# GENERAL CONCLUSIONS

## 5.1. Conclusions

This study presented the fabrication and characterization of novel PLA/CS/TCP biocomposites. Further, electrospun barrier membranes were generated using these biocomposites, and their suitability for GBR was evaluated using RAW 264.7 murine macrophages and MG63 cells. This chapter summarizes the conclusions that were drawn from this specific study and also validates some of the critical inferences from research works published on the topic of interest.

### *5.1.1. Fabrication of PLA/CS/TCP Powder Composites*

This study validated the use of a solid-state blending technique called cryomilling for the generation of powder composites made of PLA, CS, and TCP. As reported by Lim et al. (2014), a total milling time of 20 mins resulted in an obvious decrease in particle size of the constituent materials and the generation of homogeneous blends of PLA, CS, and TCP [48]. The occurrence of a homogeneous blend was confirmed using XRD. Cryomilling, being a solid-state, low-temperature blending technique eliminates problems such as the use of high temperature, need for block copolymers, solvents and the possibility of oxidation [241, 259]. Additionally, results from the pilot studies conducted also showed that homogeneous dispersion could be attained even at high loading percentages of ceramic particles. This observation was in agreement with the observations made by Lim et al. (2014) [48]. PLA and CS have very different polarities and hence achieving a compatible blend of these materials poses a challenge [47]. However, cryomilling helps decrease the particle size of the dispersed phase and enhances the attractive interaction and

thereby helps overcome the challenge of immiscibility. Moreover, when TCP is dissolved in solvents for processing, the rheological properties limit its loading, with the occurrence of aggregation and delamination [48]. In conclusion, this study reiterates the potential of cryomilling in blending materials for the generation of biocomposites.

### *5.1.2. Fabrication of PLA/CS/TCP Electrospun Membranes*

Electrospinning was used to generate barrier membranes from the cryomilled biocomposites. Electrospinning was chosen as the ideal fabrication technique based on its success in regenerative applications [292-296]. TFA was able to act as the common solvent for the purpose of fiber generation. The observations from the pilot studies revealed the inability of solvents like DCM, DMF, and chloroform to create a solution of the powder composites. This phenomenon was attributed to CS's ionic character in dissolved state and three-dimensional networks of strong hydrogen bonds [47]. Based on the visual characterization performed with SEM, it can be concluded that the decrease in PLA concentration in the powder composites resulted in thicker fibers. This observation was in agreement with the results already reported by Xu et al. (2009) [306]. Additionally, the electrospun scaffolds were able to support the growth of MG63 cells when tested under in-vitro conditions. In conclusion, this study provides evidence that electrospun membrane made out of PLA, CS, and TCP can be used for the purpose of bone regeneration.

### *5.1.3. In-vitro Studies of Powder and Electrospun Membranes*

In this study, RAW 264.7 murine macrophages were used to test the cytocompatibility of the powder composites in different concentrations (3 µl/ml, 10 µl/ml, 30 µl/ml and 50 µl/ml).

CTB® cell viability assay was used to quantitatively analyze cell viability after 24 and 48 h of placement in the culture medium. BF microscopy was used to qualitatively evaluate the morphology of the macrophages. The results from the cell viability test indicated that the generated powder composites were not toxic to cells and hence can be used to fabricate scaffolds for BTE. Then, the electrospun membranes were evaluated for their capability to support and enhance the growth of MG63 cells. The cell viability of each material was investigated using CTB® cell viability assay after 3 and 7 days from cell seeding. CARS was used to perform a live/dead assay to qualitatively analyze the ability of the scaffolds to support cell growth. The results from the live/dead assay demonstrated the viability of the scaffolds, showing more green cells (viable) than red cells (dead). The test also revealed that the cells were viable and metabolically active both after 3 and 7 days from cell seeding. The cell viability assay also indicated that the scaffolds were suitable for growing MG63 cells. The degradation results showed that the addition of CS and TCP accelerated the degradation of PLA membranes. In conclusion, this study validated the use of cryomilled PLA/CS/TCP biocomposites in scaffold-based GBR.

## 5.2. Review of Contributions

In this study, cryomilling was used to successfully generate novel biodegradable polymer-bioactive ceramic composites for the fabrication of barrier membranes to be used for the regeneration of periodontal bone defects. The results of the in vitro cell studies indicated the suitability of the biocomposites for regenerative applications.

### 5.3. Future Perspectives

There is much scope for the development of newer materials and the associated research in BTE. A few aspects that need better understanding and stronger validation are listed in this section.

Firstly, different compositions of similar composite materials need to be analyzed to identify and develop the “ideal” membrane. The validation of such materials can be strengthened by testing them in both in-vitro and in vivo environments. Another potential area of research could be the evaluation of this composite material for use in additive fabrication techniques. Bioprinting of these composites could give researchers a greater control over the morphology and dimensions of the scaffold produced. There is also need to understand the influence of cryomilling at a microscopic level and structural changes that it imparts to the materials. Moreover, the influence of cryomilling time on the cell viability of the materials needs to be explored. The influence of membrane geometry on cell viability needs investigation. A multivariate analysis needs to be performed to better understand the degradation characteristics of the barrier membranes.

## REFERENCES

[1] Eke, P. I., Dye, B. A., Wei, L., Thornton-Evans, G. O., & Genco, R. J. (2012). Prevalence of periodontitis in adults in the United States: 2009 and 2010. *Journal of dental research*, *91*(10), 914-920.

[2] Pihlstrom, B. L., Michalowicz, B. S., & Johnson, N. W. (2005). Periodontal diseases. *The Lancet*, *366*(9499), 1809-1820.

[3] Nakashima, M., & Reddi, A. H. (2003). The application of bone morphogenetic proteins to dental tissue engineering. *Nature biotechnology*, *21*(9), 1025-1032.

[4] Nanci, A., & Bosshardt, D. D. (2006). Structure of periodontal tissues in health and disease. *Periodontology 2000*, *40*(1), 11-28.

[5] Bascones-Martínez, A., Muñoz-Corcuera, M., Noronha, S., Mota, P., Bascones-Ilundain, C., & Campo-Trapero, J. (2009). Host defence mechanisms against bacterial aggression in periodontal disease: Basic mechanisms. *Med Oral Patol Oral Cir Bucal*, *14*(12), e680-5.

[6] Zhang, L., Henson, B. S., Camargo, P. M., & Wong, D. T. (2009). The clinical value of salivary biomarkers for periodontal disease. *Periodontology 2000*, *51*(1), 25-37.

[7] Periodontal Treatments and Procedures. Retrieved from https://www.perio.org/consumer/treatments-procedures

[8] Fiorellini, J. P., & Nevins, M. L. (2003). Localized ridge augmentation/preservation. A systematic review. *Annals of Periodontology*, *8*(1), 321-327.

[9] Chiapasco, M., & Zaniboni, M. (2009). Clinical outcomes of GBR procedures to correct peri-implant dehiscences and fenestrations: a systematic review. *Clinical Oral Implants Research*, *20*(s4), 113-123.

[10] Dahlin, C., Linde, A., Gottlow, J., & Nyman, S. (1988). Healing of bone defects by guided tissue regeneration. *Plastic and reconstructive surgery*, *81*(5), 672-676.

[11] Bottino, M. C., Thomas, V., Schmidt, G., Vohra, Y. K., Chu, T. M. G., Kowolik, M. J., & Janowski, G. M. (2012). Recent advances in the development of GTR/GBR membranes for periodontal regeneration—a materials perspective. *Dental materials*, *28*(7), 703-721.

[12] Carmen, J., Burger, S. R., McCaman, M., & Rowley, J. A. (2012). Developing assays to address identity, potency, purity and safety: cell characterization in cell therapy process development. *Regenerative medicine*, *7*(1), 85-100.

[13] Purser, M. F., Wysk, R. A., Shirwaiker, R. A., Harrysson, O., Cohen, P. H., Jackson, J. D. & Atala, A. J. (2012). Regenerative Medicine Manufacturing—A Systems Engineering Perspective.

*In New Directions in Informatics, Optimization, Logistics, and Production* (pp. 50-58). INFORMS.

[14] Retzepi, M., & Donos, N. (2010). Guided bone regeneration: biological principle and therapeutic applications. *Clinical oral implants research*, *21*(6), 567-576.

[15] Scantlebury, T. V. (1993). 1982-1992: A Decade of Technology Development for Guided Tissue Regeneration*. *Journal of periodontology*, *64*(11s), 1129-1137.

[16] Needleman, I., Tucker, R., Giedrys-Leeper, E., & Worthington, H. (2002). A systematic review of guided tissue regeneration for periodontal infrabony defects. *Journal of periodontal research*, *37*(5), 380-388.

[17] Murphy, K. G., & Gunsolley, J. C. (2003). Guided tissue regeneration for the treatment of periodontal intrabony and furcation defects. A systematic review. *Annals of Periodontology*, *8*(1), 266-302.

[18] Wang, J., Wang, L., Zhou, Z., Lai, H., Xu, P., Liao, L., & Wei, J. (2016). Biodegradable polymer membranes applied in guided bone/tissue regeneration: A review. *Polymers*, *8*(4), 115.

[19] Bunyaratavej, P., & Wang, H. L. (2001). Collagen membranes: a review. *Journal of periodontology*, *72*(2), 215-229.

[20] Hutmacher, D., Hürzeler, M. B., & Schliephake, H. (1996). A review of material properties of biodegradable and bioresorbable polymers and devices for GTR and GBR applications. *International Journal of Oral & Maxillofacial Implants*, *11*(5).

[21] Mellonig, J. T., & Nevins, M. (1995). Guided bone regeneration of bone defects associated with implants: an evidence-based outcome assessment. *International Journal of Periodontics & Restorative Dentistry*, *15*(2).

[22] Bartee, B. K. (1995). A membrane and graft technique for ridge maintenance using high-density polytetrafluoroethylene membrane (n-PTFE) and hydroxylapatite: report of four cases. *Texas dental journal*, *112*(5), 7-9.

[23] Bartee, B. K. (1995). The use of high-density polytetrafluoroethylene membrane to treat osseous defects: clinical reports. *Implant dentistry*, *4*(1), 21-31.

[24] Jovanovic, S. A., & Nevins, M. (1995). Bone formation utilizing titanium-reinforced barrier membranes. *International Journal of Periodontics & Restorative Dentistry*, *15*(1).

[25] von Arx, T., Hardt, N., & Wallkamm, B. (1996). The TIME technique: a new method for localized alveolar ridge augmentation prior to placement of dental implants. *International Journal of Oral & Maxillofacial Implants*, *11*(3).

[26] Buser, D., Dula, K., Hirt, H. P., & Schenk, R. K. (1996). Lateral ridge augmentation using autografts and barrier membranes: a clinical study with 40 partially edentulous patients. *Journal of Oral and Maxillofacial Surgery*, *54*(4), 420-432.

[27] Buser, D., Dula, K., Belser, U., Hirt, H. P., & Berthold, H. (1993). Localized Ridge Augmentation Using Guided Bone Regeneration. I. Surgical Procedure in the Maxilla. *International Journal of Periodontics & Restorative Dentistry*, *13*(1).

[28] Busei, D., Dula, K., Belser, U. C., Hirt, H. P., & Berthold, H. (1995). Localized ridge augmentation using guided bone regeneration. II. Surgical procedure in the mandible. *International Journal of Periodontics & Restorative Dentistry*, *15*(1).

[29] Verardi, S., & Simion, M. (2007). Management of the exposure of e-PTFE membranes in guided bone regeneration. *Practical procedures & aesthetic dentistry: PPAD*, *19*(2), 111-117.

[30] Jung, R. E., Fenner, N., Hämmerle, C. H., & Zitzmann, N. U. (2013). Long-term outcome of implants placed with guided bone regeneration (GBR) using resorbable and non-resorbable membranes after 12–14 years. *Clinical Oral Implants Research*, *24*(10), 1065-1073.

[31] Kuo, S. M., Chang, S. J., Chen, T. W., & Kuan, T. C. (2006). Guided tissue regeneration for using a chitosan membrane: an experimental study in rats. *Journal of Biomedical Materials Research Part A*, *76*(2), 408-415.

[32] Lee, E. J., Shin, D. S., Kim, H. E., Kim, H. W., Koh, Y. H., & Jang, J. H. (2009). Membrane of hybrid chitosan–silica xerogel for guided bone regeneration. *Biomaterials*, *30*(5), 743-750.

[33] Shin, S. Y., Park, H. N., Kim, K. H., Lee, M. H., Choi, Y. S., Park, Y. J., ... & Lee, S. J. (2005). Biological evaluation of chitosan nanofiber membrane for guided bone regeneration. *Journal of periodontology*, *76*(10), 1778-1784.

[34] Sheikh, Z., Qureshi, J., Alshahrani, A. M., Nassar, H., Ikeda, Y., Glogauer, M., & Ganss, B. (2017). Collagen based barrier membranes for periodontal guided. *Odontology*, *105*(1), 1-12.

[35] Miller, N., Penaud, J., Foliguet, B., Membre, H., Ambrosini, P., & Plombas, M. (1996). Resorption rates of 2 commercially available bioresorbable membranes. *Journal of clinical periodontology*, *23*(12), 1051-1059.

[36] Araujo, M. G., Berglundh, T., & Lindhe, J. (1998). GTR treatment of degree III furcation defects with 2 different resorbable barriers: An experimental study in dogs. *Journal of clinical periodontology*, *25*(3), 253-259.

[37] Yang, F., Both, S. K., Yang, X., Walboomers, X. F., & Jansen, J. A. (2009). Development of an electrospun nano-apatite/PCL composite membrane for GTR/GBR application. *Acta biomaterialia*, *5*(9), 3295-3304

[38] Hua, N., Ti, V. L., & Xu, Y. (2014). Biodegradable effect of PLGA membrane in alveolar bone regeneration on beagle dog. *Cell biochemistry and biophysics*, *70*(2), 1051-1055.

[39] Oh, S. H., Kim, J. H., Kim, J. M., & Lee, J. H. (2006). Asymmetrically porous PLGA/Pluronic F127 membrane for effective guided bone regeneration. *Journal of Biomaterials Science, Polymer Edition*, *17*(12), 1375-1387.

[40] Won, J. Y., Park, C. Y., Bae, J. H., Ahn, G., Kim, C., Lim, D. H., ... & Huh, J. B. (2016). Evaluation of 3D printed PCL/PLGA/β-TCP versus collagen membranes for guided bone regeneration in a beagle implant model. *Biomedical Materials*, *11*(5), 055013.

[41] Li, J., Zuo, Y., Cheng, X., Yang, W., Wang, H., & Li, Y. (2009). Preparation and characterization of nano-hydroxyapatite/polyamide 66 composite GBR membrane with asymmetric porous structure. *Journal of Materials Science: Materials in Medicine*, *20*(5), 1031-1038.

[42] Kikuchi, M., Koyama, Y., Takakuda, K., Miyairi, H., Shirahama, N., & Tanaka, J. (2002). In vitro change in mechanical strength of β-tricalcium phosphate/copolymerized poly-L-lactide composites and their application for guided bone regeneration. *Journal of biomedical materials research*, *62*(2), 265-272.

[43] Seol, Y. J., Lee, J. Y., Park, Y. J., Lee, Y. M., Rhyu, I. C., Lee, S. J., ... & Chung, C. P. (2004). Chitosan sponges as tissue engineering scaffolds for bone formation. *Biotechnology letters*, *26*(13), 1037-1041.

[44] Aranaz, I., Mengíbar, M., Harris, R., Paños, I., Miralles, B., Acosta, N. & Heras, Á. (2009). Functional characterization of chitin and chitosan. *Current Chemical Biology*, *3*(2), 203-230.

[45] Şenel, S., & McClure, S. J. (2004). Potential applications of chitosan in veterinary medicine. *Advanced drug delivery reviews*, *56*(10), 1467-1480.

[46] Irigaray, J., Oudadesse, H., Blondiaux, G., & Collangettes, D. (1993). Kinetics of the diffusion of some elements evaluated by neutron activation in a coral implanted in vivo. *Journal of radioanalytical and nuclear chemistry*, *169*(2), 339-346.

[47] Geng, X., Kwon, O. H., & Jang, J. (2005). Electrospinning of chitosan dissolved in concentrated acetic acid solution. *Biomaterials*, *26*(27), 5427-5432.

[48] Lim, J., Chong, M. S. K., Chan, J. K. Y., & Teoh, S. H. (2014). Polymer Powder Processing of Cryomilled Polycaprolactone for Solvent-Free Generation of Homogeneous Bioactive Tissue Engineering Scaffolds. *Small*, *10*(12), 2495-2502

[49] Albandar, J. M. (2005). Epidemiology and risk factors of periodontal diseases. *Dental Clinics of North America*, *49*(3), 517-532.

[50] Khalili, J. (2007). Periodontal disease: an overview for medical practitioners. *Likars' ka sprava/Ministerstvo okhorony zdorov'ia Ukrainy*, (3-4), 10-21.

[51] Haynes, D. R. (2006). Emerging and future therapies for the treatment of bone loss associated with chronic inflammation. *Inflammopharmacology*, *14*(5-6), 193-197.

[52] Armitage, G. C. (1999). Development of a classification system for periodontal diseases and conditions. *Annals of periodontology*, *4*(1), 1-6.

[53] Baldi, D., Izzotti, A., Bonica, P., Pera, P., & Pulliero, A. (2009). Degenerative periodontal-diseases and oral osteonecrosis: the role of gene-environment interactions. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, *667*(1), 118-131.

[54] Armitage, G. C., & Cullinan, M. P. (2010). Comparison of the clinical features of chronic and aggressive periodontitis. *Periodontology 2000*, *53*(1), 12-27.

[55] Huang, D. M., Ling, J. Q., Fu, C. H., Luo, H. X., & Zhou, X. D. (2005). Colonization relationship between Porphyromonas gingivalis and Bacteroides forsythus in the infected root canals with chronic apical periodontitis. *Shanghai kou qiang yi xue= Shanghai journal of stomatology*, *14*(5), 531-535.

[56] Lovegrove, J. M. (2003). Dental plaque revisited: bacteria associated with periodontal disease. *Journal of the New Zealand Society of Periodontology*, (87), 7-21.

[57] Tanabe, S. I., Bodet, C., & Grenier, D. (2007). Peptostreptococcus micros cell wall elicits a pro-inflammatory response in human macrophages. *Journal of endotoxin research*, *13*(4), 219-226.

[58] Saito, Y., Fujii, R., Nakagawa, K. I., Kuramitsu, H. K., Okuda, K., & Ishihara, K. (2008). Stimulation of Fusobacterium nucleatum biofilm formation by Porphyromonas gingivalis. *Oral microbiology and immunology*, *23*(1), 1-6.

[59] Zini, A., Sgan-Cohen, H. D., & Marcenes, W. (2011). Socio-economic position, smoking, and plaque: a pathway to severe chronic periodontitis. *Journal of clinical periodontology*, *38*(3), 229-235.

[60] Campus, G., Salem, A., Uzzau, S., Baldoni, E., & Tonolo, G. (2005). Diabetes and periodontal disease: a case-control study. *Journal of periodontology*, *76*(3), 418-425.

[61] Graves, D. T., Al-Mashat, H., & Liu, R. (2004). Evidence that diabetes mellitus aggravates periodontal diseases and modifies the response to an oral pathogen in animal models. *Compendium of continuing education in dentistry (Jamesburg, NJ: 1995)*, *25*(7 Suppl 1), 38-45.

[62] Meng, H. X. (2007). Association between periodontitis and diabetes mellitus. *Beijing da xue xue bao. Yi xue ban= Journal of Peking University. Health sciences*, *39*(1), 18-20.

[63] Stegeman, C. A. (2005). Oral manifestations of diabetes. *Home Healthcare Now*, *23*(4), 233-240.

[64] Kamil, W., Al Habashneh, R., Khader, Y., Al Bayati, L., & Taani, D. (2011). Effects of nonsurgical periodontal therapy on C-reactive protein and serum lipids in Jordanian adults with advanced periodontitis. *Journal of periodontal research*, *46*(5), 616-621.

[65] Izumi, A., Yoshihara, A., Hirotomi, T., & Miyazaki, H. (2009). The relationship between serum lipids and periodontitis in elderly non-smokers. *Journal of periodontology*, *80*(5), 740-748.

[66] Merchant, A. T., PITIPHAT, W., AHMED, B., KAWACHI, I., & JOSHIPURA, K. (2003). A prospective study of social support, anger expression and risk of periodontitis in men. *The Journal of the American Dental Association*, *134*(12), 1591-1596.

[67] Dalla Vecchia, C. F., Susin, C., Rösing, C. K., Oppermann, R. V., & Albandar, J. M. (2005). Overweight and obesity as risk indicators for periodontitis in adults. *Journal of periodontology*, *76*(10), 1721-1728.

[68] Pischon, N., Heng, N., Bernimoulin, J. P., Kleber, B. M., Willich, S. N., & Pischon, T. (2007). Obesity, inflammation, and periodontal disease. *Journal of dental research*, *86*(5), 400-409.

[69] Shimazaki, Y., Egami, Y., Matsubara, T., Koike, G., Akifusa, S., Jingu, S., & Yamashita, Y. (2010). Relationship between obesity and physical fitness and periodontitis. *Journal of periodontology*, *81*(8), 1124-1131.

[70] Al Habashneh, R., Alchalabi, H. A., Khader, Y. S., Hazza'a, A. M., Odat, Z., & Johnson, G. K. (2010). Association between periodontal disease and osteoporosis in postmenopausal women in Jordan. *Journal of periodontology*, *81*(11), 1613-1621.

[71] Tayeb, Y., Goultschin, J., Fogel, M., & Schwartz, Z. (2003). The relationship between osteoporosis, osteopenia and periodontitis. *Refu'at ha-peh veha-shinayim (1993)*, *20*(1), 8-22.

[72] Wactawski-Wende, J., Grossi, S. G., Trevisan, M., Genco, R. J., Tezal, M., Dunford, R. G., & Hreshchyshyn, M. M. (1996). The role of osteopenia in oral bone loss and periodontal disease. *Journal of Periodontology*, *67*(10s), 1076-1084.

[73] AlJehani, Y. A. (2014). Risk factors of periodontal disease: review of the literature. *International journal of dentistry*, *2014*.

[74] Haffajee, A. D., & Socransky, S. S. (1994). Microbial etiological agents of destructive periodontal diseases. *Periodontology 2000*, *5*(1), 78-111.

[75] Hienz, S. A., Paliwal, S., & Ivanovski, S. (2015). Mechanisms of bone resorption in periodontitis. *Journal of immunology research, 2015.*

[76] Oates, T. W., & Cochran, D. L. (1995). Bone cell interactions and regulation by inflammatory mediators. *Current opinion in periodontology*, *3*, 34-44.

[77] McCauley, L. K., & Nohutcu, R. M. (2002). Mediators of periodontal osseous destruction and remodeling: principles and implications for diagnosis and therapy. *Journal of periodontology*, *73*(11), 1377-1391.

[78] Teitelbaum, S. L. (2007). Osteoclasts: what do they do and how do they do it? The *American journal of pathology*, *170*(2), 427-435.

[79] Yasuda, H., Shima, N., Nakagawa, N., Yamaguchi, K., Kinosaki, M., Mochizuki, S. I., & Tsuda, E. (1998). Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proceedings of the National Academy of Sciences*, *95*(7), 3597-3602.

[80] Nagasawa, T., Kiji, M., Yashiro, R., Hormdee, D., Lu, H., Kunze, M., & Nitta, H. (2007). Roles of receptor activator of nuclear factor-κB ligand (RANKL) and osteoprotegerin in periodontal health and disease. *Periodontology 2000*, *43*(1), 65-84.

[81] MacDonald, B. R. (1986). Parathyroid hormone, prostaglandins and bone resorption. In *Nutritional Problems and Education: Selected Topics* (pp. 163-201). Karger Publishers.

[82] Katz, B. G. (2013). *Guided tissue regeneration: background to current indications and applications.* Retrieved from http://www.perioimplantadvisory.com/articles/2013/11/guided-tissue-regeneration-background-to-current-indications-and-applications.html

[83] Mason, C., & Dunnill, P. (2008). A brief definition of regenerative medicine.

[84] Lekovic, V., Kenney, E. B., Weinlaender, M., Han, T., Klokkevold, P., Nedic, M., & Orsini, M. (1997). A bone regenerative approach to alveolar ridge maintenance following tooth extraction. Report of 10 cases. *Journal of periodontology*, *68*(6), 563-570.

[85] Lekovic, V., Klokkevold, P. R., Kenney, E. B., Dimitrijelic, B., Nedic, M., & Weinlaender, M. (1998). Histologic evaluation of guided tissue regeneration using 4 barrier membranes: a comparative furcation study in dogs. *Journal of periodontology*, *69*(1), 54-61.

[86] Van der Weijden, F., Dell'Acqua, F., & Slot, D. E. (2009). Alveolar bone dimensional changes of post-extraction sockets in humans: a systematic review. *Journal of clinical periodontology*, *36*(12), 1048-1058.

[87] Cardaropoli, G., Araujo, M., & Lindhe, J. (2003). Dynamics of bone tissue formation in tooth extraction sites. *Journal of clinical periodontology*, *30*(9), 809-818.

[88] Schropp, L., Wenzel, A., Kostopoulos, L., & Karring, T. (2003). Bone healing and soft tissue contour changes following single-tooth extraction: a clinical and radiographic 12-month prospective study. *International Journal of Periodontics and Restorative Dentistry, 23*(4), 313-324.

[89] McAllister, B. S., & Haghighat, K. (2007). Bone augmentation techniques. *Journal of periodontology, 78*(3), 377-396.

[90] Seibert, J., & Nyman, S. (1990). Localized ridge augmentation in dogs: a pilot study using membranes and hydroxyapatite. *Journal of periodontology, 61*(3), 157-165.

[91] Smukler, H., Barboza, E. P., & Burliss, C. (1995). A new approach to regeneration of surgically reduced alveolar ridges in dogs: a clinical and histologic study. *International Journal of Oral & Maxillofacial Implants, 10*(5).

[92] Lang, N. P., Hämmerle, C. H. F., Brägger, U., Lehmann, B., & Nyman, S. R. (1994). Guided tissue regeneration in jawbone defects prior to implant placement. *Clinical oral implants research, 5*(2), 92-97.

[93] Warrer, K., Gotfredsen, K., Hjsrting-hansen, E., & Karring, T. (1991). Guided tissue regeneration ensures osseointegration of dental implants placed into extraction sockets. An experimental study in monkeys. *Clinical Oral Implants Research, 2*(4), 166-171.

[94] Dahlin, C., Andersson, L., & Linde, A. (1991). Bone augmentation at fenestrated implants by an osteopromotive membrane technique. A controlled clinical study. *Clinical Oral Implants Research, 2*(4), 159-165.

[95] Jovanovic, S. A., Spiekermann, H., & Richter, E. J. (1992). Bone regeneration around titanium dental implants in dehisced defect sites: a clinical study. *International Journal of Oral & Maxillofacial Implants, 7*(2).

[96] Lundgren, D., Sennerby, L., Falk, H., Friberg, B., & Nyman, S. (1994). The use of a new bioresorbable barrier for guided bone regeneration in connection with implant installation. Case reports. *Clinical oral implants research, 5*(3), 177-184.

[97] Nobréus, N., Attström, R., & Linde, A. (1997). Guided bone regeneration in dental implant treatment using a bioabsorbable membrane. *Clinical oral implants research, 8*(1), 10-17.

[98] Kostopoulos, L., & Karring, T. (1994). Guided bone regeneration in mandibular defects in rats using a bioresorbable polymer. *Clinical oral implants research, 5*(2), 66-74.

[99] Retzepi, M., Lewis, M. P., & Donos, N. (2010). Effect of diabetes and metabolic control on de novo bone formation following guided bone regeneration. *Clinical oral implants research, 21*(1), 71-79.

[100] Campbell, J. B., Bassett, C. A. L., Girado, J. M., Seymour, R. J., & Rossi, J. P. (1956). Application of Monomolecular Filter Tubes in Bridging Gaps in Peripheral Nerves and for Prevention of Neuroma Formation* A Preliminary Report. *Journal of neurosurgery, 13*(6), 635-637.

[101] Ashley, F. L., Stone, R. S., Alonso-Artieda, M., Syverud, J. M., Edwards, J. W., Sloan, R. F., & Mooney, S. A. (1959). Experimental and clinical studies on the application of monomolecular cellulose filter, tubes to create artificial tendon sheaths in digits. *Plastic and Reconstructive Surgery, 23*(5), 526-534.

[102] Hurley, L. A., Stinchfield, F. E., Bassett, C. A. L., & Lyon, W. H. (1959). The Role of Soft Tissues in Osteogenesis: An Experimental Study of Canine Spine Fusions. *JBJS, 41*(7), 1243-1266.

[103] Rüedi, T. P., & Bassett, C. A. L. (1967). Repair and remodeling in Millipore-isolated defects in cortical bone. *Cells Tissues Organs, 68*(4), 509-531.

[104] Kahnberg, K. E. (1979). Restoration of mandibular jaw defects in the rabbit by subperiosteally implanted Teflon® mantle leaf. *International journal of oral surgery, 8*(6), 449-456.

[105] Melcher, A. H. (1969). Role of the periosteum in repair of wounds of the parietal bone of the rat. *Archives of Oral Biology, 14*(9), 1101IN23-1109IN25.

[106] Moses, O., Pitaru, S., Artzi, Z., & Nemcovsky, C. E. (2005). Healing of dehiscence-type defects in implants placed together with different barrier membranes: a comparative clinical study. *Clinical oral implants research, 16*(2), 210-219.

[107] Lee, J. Y., Lee, J., & Kim, Y. K. (2013). Comparative analysis of guided bone regeneration using autogenous tooth bone graft material with and without resorbable membrane. *Journal of Dental Sciences, 8*(3), 281-286.

[108] Beitlitum, I., Artzi, Z., & Nemcovsky, C. E. (2010). Clinical evaluation of particulate allogeneic with and without autogenous bone grafts and resorbable collagen membranes for bone augmentation of atrophic alveolar ridges. *Clinical oral implants research, 21*(11), 1242-1250.

[109] Saravanan, S., Leena, R. S., & Selvamurugan, N. (2016). Chitosan based biocomposite scaffolds for bone tissue engineering. *International journal of biological macromolecules, 93*, 1354-1365.

[110] Piattelli, A., Scarano, A., Russo, P., & Matarasso, S. (1996). Evaluation of guided bone regeneration in rabbit tibia using bioresorbable and non-resorbable membranes. *Biomaterials, 17*(8), 791-796.

[111] Sculean, A., Nikolidakis, D., & Schwarz, F. (2008). Regeneration of periodontal tissues: combinations of barrier membranes and grafting materials–biological foundation and preclinical evidence: a systematic review. *Journal of Clinical Periodontology, 35*(s8), 106-116.

[112] Dimitriou, R., Mataliotakis, G. I., Calori, G. M., & Giannoudis, P. V. (2012). The role of barrier membranes for guided bone regeneration and restoration of large bone defects: current experimental and clinical evidence. *BMC medicine*, *10*(1), 81.

[113] Sowmya, S., Bumgardener, J. D., Chennazhi, K. P., Nair, S. V., & Jayakumar, R. (2013). Role of nanostructured biopolymers and bioceramics in enamel, dentin and periodontal tissue regeneration. *Progress in Polymer Science, 38*(10), 1748-1772.

[114] Aaboe, M., Pinholt, E. M., & Hjorting-Hansen, E. (1995). Healing of experimentally created defects: a review. *British Journal of Oral and Maxillofacial Surgery, 33*(5), 312-318.

[115] Schmidmaier, G., Baehr, K., Mohr, S., Kretschmar, M., Beck, S., & Wildemann, B. (2006). Biodegradable polylactide membranes for bone defect coverage: biocompatibility testing, radiological and histological evaluation in a sheep model. *Clinical oral implants research, 17*(4), 439-444.

[116] Gielkens, P. F., Schortinghuis, J., De Jong, J. R., Raghoebar, G. M., Stegenga, B., & Bos, R. R. (2008). Vivosorb®, Bio-Gide®, and Gore-Tex® as barrier membranes in rat mandibular defects: an evaluation by microradiography and micro-CT. *Clinical oral implants research, 19*(5), 516-521.

[117] Fujihara, K., Kotaki, M., & Ramakrishna, S. (2005). Guided bone regeneration membrane made of polycaprolactone/calcium carbonate composite nano-fibers. *Biomaterials, 26*(19), 4139-4147.

[118] Kozlovsky, A., Aboodi, G., Moses, O., Tal, H., Artzi, Z., Weinreb, M., & Nemcovsky, C. E. (2009). Bio-degradation of a resorbable collagen membrane (Bio-Gide®) applied in a double-layer technique in rats. *Clinical oral implants research*, *20*(10), 1116-1123.

[119] Felipe, M. E. M., Andrade, P. F., Grisi, M. F., Souza, S. L., Taba Jr, M., Palioto, D. B., & Novaes Jr, A. B. (2007). Comparison of two surgical procedures for use of the acellular dermal matrix graft in the treatment of gingival recessions: a randomized controlled clinical study. *Journal of periodontology*, *78*(7), 1209-1217.

[120] Santos, A., Goumenos, G., & Pascual, A. (2005). Management of gingival recession by the use of an acellular dermal graft material: a 12-case series. *Journal of periodontology*, *76*(11), 1982-1990.

[121] Sung, H. J., Meredith, C., Johnson, C., & Galis, Z. S. (2004). The effect of scaffold degradation rate on three-dimensional cell growth and angiogenesis. *Biomaterials, 25*(26), 5735-5742.

[122] Behring, J., Junker, R., Walboomers, X. F., Chessnut, B., & Jansen, J. A. (2008). Toward guided tissue and bone regeneration: morphology, attachment, proliferation, and migration of cells cultured on collagen barrier membranes. A systematic review. *Odontology*, *96*(1), 1-11.

[123] Bhrany, A. D., Lien, C. J., Beckstead, B. L., Futran, N. D., Muni, N. H., Giachelli, C. M., & Ratner, B. D. (2008). Crosslinking of an oesophagus acellular matrix tissue scaffold. *Journal of tissue engineering and regenerative medicine*, *2*(6), 365-372.

[124] Sundararaghavan, H. G., Monteiro, G. A., Lapin, N. A., Chabal, Y. J., Miksan, J. R., & Shreiber, D. I. (2008). Genipin-induced changes in collagen gels: Correlation of mechanical properties to fluorescence. *Journal of biomedical materials research Part A*, *87*(2), 308-320.

[125] Sung, H. W., Chang, Y., Chiu, C. T., Chen, C. N., & Liang, H. C. (1999). Crosslinking characteristics and mechanical properties of a bovine pericardium fixed with a naturally occurring crosslinking agent. *Journal of biomedical materials research*, *47*(2), 116-126.

[126] Kikuchi, M., Koyama, Y., Yamada, T., Imamura, Y., Okada, T., Shirahama, N., & Tanaka, J. (2004). Development of guided bone regeneration membrane composed of β-tricalcium phosphate and poly (L-lactide-co-glycolide-co-ε-caprolactone) composites. *Biomaterials*, *25*(28), 5979-5986.

[127] Liao, S., Wang, W., Uo, M., Ohkawa, S., Akasaka, T., Tamura, K., & Watari, F. (2005). A three-layered nano-carbonated hydroxyapatite/collagen/PLGA composite membrane for guided tissue regeneration. *Biomaterials*, *26*(36), 7564-7571.

[128] Liao, S., Watari, F., Zhu, Y., Uo, M., Akasaka, T., Wang, W., & Cui, F. (2007). The degradation of the three layered nano-carbonated hydroxyapatite/collagen/PLGA composite membrane in vitro. *Dental Materials*, *23*(9), 1120-1128.

[129] Klinger, A., Asad, R., Shapira, L., & Zubery, Y. (2010). In vivo degradation of collagen barrier membranes exposed to the oral cavity. *Clinical oral implants research*, *21*(8), 873-876.

[130] Thoma, D. S., Villar, C. C., Cochran, D. L., Hämmerle, C. H., & Jung, R. E. (2012). Tissue integration of collagen-based matrices: an experimental study in mice. *Clinical oral implants research*, *23*(12), 1333-1339.

[131] Owens, K. W., & Yukna, R. A. (2001). Collagen membrane resorption in dogs: a comparative study. *Implant dentistry*, *10*(1), 49-58.

[132] Rothamel, D., Schwarz, F., Sager, M., Herten, M., Sculean, A., & Becker, J. (2005). Biodegradation of differently cross-linked collagen membranes: an experimental study in the rat. *Clinical oral implants research*, *16*(3), 369-378.

[133] Rothamel, D., Schwarz, F., Fienitz, T., Smeets, R., Dreiseidler, T., Ritter, L., & Zöller, J. (2012). Biocompatibility and biodegradation of a native porcine pericardium membrane: results of in vitro and in vivo examinations. *International Journal of Oral and Maxillofacial Implants, 27*(1), 146.

[134] Benic, G. I., & Hämmerle, C. H. (2014). Horizontal bone augmentation by means of guided bone regeneration. *Periodontology 2000, 66*(1), 13-40.

[135] Milella, E., Barra, G., Ramires, P. A., Leo, G., Aversa, P., & Romito, A. (2001). Poly (L-lactide) acid/alginate composite membranes for guided tissue regeneration. *Journal of biomedical materials research, 57*(2), 248-257.

[136] Gentile, P., Chiono, V., Tonda-Turo, C., Ferreira, A. M., & Ciardelli, G. (2011). Polymeric membranes for guided bone regeneration. *Biotechnology Journal, 6*(10), 1187-1197.

[137] Wiltfang, J., Merten, H. A., & Peters, J. H. (1998). Comparative study of guided bone regeneration using absorbable and permanent barrier membranes: a histologic report. *International Journal of Oral and Maxillofacial Implants, 13*(3), 416-421.

[138] Polimeni, G., Albandar, J. M., & Wikesjö, U. M. (2005). Prognostic factors for alveolar regeneration: effect of space provision. *Journal of clinical periodontology, 32*(9), 951-954.

[139] Sung, H. W., Huang, R. N., Huang, L. L., & Tsai, C. C. (1999). In vitro evaluation of cytotoxicity of a naturally occurring cross-linking reagent for biological tissue fixation. *Journal of Biomaterials Science, Polymer Edition, 10*(1), 63-78.

[140] Sung, H. W., Chang, Y., Chiu, C. T., Chen, C. N., & Liang, H. C. (1999). Mechanical properties of a porcine aortic valve fixed with a naturally occurring crosslinking agent. *Biomaterials, 20*(19), 1759-1772.

[141] Veríssimo, D. M., Leitão, R. F. C., Ribeiro, R. A., Figueiró, S. D., Sombra, A. S. B., Góes, J. C., & Brito, G. A. C. (2010). Polyanionic collagen membranes for guided tissue regeneration: Effect of progressive glutaraldehyde cross-linking on biocompatibility and degradation. Acta *Biomaterialia, 6*(10), 4011-4018.

[142] Schwarz, F., Rothamel, D., Herten, M., Sager, M., & Becker, J. (2006). Angiogenesis pattern of native and cross-linked collagen membranes: an immunohistochemical study in the rat. *Clinical Oral Implants Research, 17*(4), 403-409.

[143] Cha, J. K., Joo, M. J., Yoon, S., Lee, J. S., Choi, S. H., & Jung, U. W. (2016). Sequential healing of onlay bone grafts using combining biomaterials with cross-linked collagen in dogs. *Clinical oral implants research.*

[144] Annen, B. M., Ramel, C. F., Hammerle, C. H. F., & Jung, R. E. (2011). Use of a new cross-linked collagen membrane for the treatment of peri-implant dehiscence defects: a randomised controlled double-blinded clinical trial. *European journal of oral implantology, 4*(2).

[145] Jayakumar, R., Prabaharan, M., Kumar, P. S., Nair, S. V., & Tamura, H. (2011). Biomaterials based on chitin and chitosan in wound dressing applications. *Biotechnology advances, 29*(3), 322-337.

[146] Kim, I. Y., Seo, S. J., Moon, H. S., Yoo, M. K., Park, I. Y., Kim, B. C., & Cho, C. S. (2008). Chitosan and its derivatives for tissue engineering applications. *Biotechnology advances, 26*(1), 1-21.

[147] Bhattarai, N., Edmondson, D., Veiseh, O., Matsen, F. A., & Zhang, M. (2005). Electrospun chitosan-based nanofibers and their cellular compatibility. *Biomaterials, 26*(31), 6176-6184.

[148] Lee, K. Y., Jeong, L., Kang, Y. O., Lee, S. J., & Park, W. H. (2009). Electrospinning of polysaccharides for regenerative medicine. *Advanced drug delivery reviews, 61*(12), 1020-1032.

[149] Muzzarelli, R. A. A., Mattioli-Belmonte, M., Tietz, C., Biagini, R., Ferioli, G., Brunelli, M. A., & Biagini, G. (1994). Stimulatory effect on bone formation exerted by a modified chitosan. *Biomaterials, 15*(13), 1075-1081.

[150] Mano, J. F., Silva, G. A., Azevedo, H. S., Malafaya, P. B., Sousa, R. A., Silva, S. S., & Neves, N. M. (2007). Natural origin biodegradable systems in tissue engineering and regenerative medicine: present status and some moving trends. *Journal of the Royal Society Interface, 4*(17), 999-1030.

[151] Di Martino, A., Sittinger, M., & Risbud, M. V. (2005). Chitosan: a versatile biopolymer for orthopaedic tissue-engineering. *Biomaterials, 26*(30), 5983-5990.

[152] Zhang, Y., Ni, M., Zhang, M., & Ratner, B. (2003). Calcium phosphate—chitosan composite scaffolds for bone tissue engineering. *Tissue engineering, 9*(2), 337-345.

[153] Sowjanya, J. A., Singh, J., Mohita, T., Sarvanan, S., Moorthi, A., Srinivasan, N., & Selvamurugan, N. (2013). Biocomposite scaffolds containing chitosan/alginate/nano-silica for bone tissue engineering. *Colloids and Surfaces B: Biointerfaces, 109*, 294-300.

[154] Bavariya, A. J., Andrew Norowski, P., Mark Anderson, K., Adatrow, P. C., Garcia-Godoy, F., Stein, S. H., & Bumgardner, J. D. (2014). Evaluation of biocompatibility and degradation of chitosan nanofiber membrane crosslinked with genipin. *Journal of Biomedical Materials Research Part B: Applied Biomaterials, 102*(5), 1084-1092.

[155] Mi, F. L., Tan, Y. C., Liang, H. C., Huang, R. N., & Sung, H. W. (2001). In vitro evaluation of a chitosan membrane cross-linked with genipin. *Journal of Biomaterials Science, Polymer Edition, 12*(8), 835-850.

[156] Kaya, M., Baran, T., Erdoğan, S., Menteş, A., Özüsağlam, M. A., & Çakmak, Y. S. (2014). Physicochemical comparison of chitin and chitosan obtained from larvae and adult Colorado potato beetle (Leptinotarsa decemlineata). *Materials Science and Engineering: C, 45*, 72-81.

[157] Xu, C., Lei, C., Meng, L., Wang, C., & Song, Y. (2012). Chitosan as a barrier membrane material in periodontal tissue regeneration. *Journal of Biomedical Materials Research Part B: Applied Biomaterials, 100*(5), 1435-1443.

[158] Mota, J., Yu, N., Caridade, S. G., Luz, G. M., Gomes, M. E., Reis, R. L., ... & Mano, J. F. (2012). Chitosan/bioactive glass nanoparticle composite membranes for periodontal regeneration. *Acta Biomaterialia, 8*(11), 4173-4180.

[159] Dash, M., Chiellini, F., Ottenbrite, R. M., & Chiellini, E. (2011). Chitosan—A versatile semi-synthetic polymer in biomedical applications. *Progress in polymer science, 36*(8), 981-1014.

[160] Qasim, S. B., Delaine-Smith, R. M., Fey, T., Rawlinson, A., & Rehman, I. U. (2015). Freeze gelated porous membranes for periodontal tissue regeneration. *Acta biomaterialia, 23*, 317-328.

[161] Mogoşanu, G. D., & Grumezescu, A. M. (2014). Natural and synthetic polymers for wounds and burns dressing. *International journal of pharmaceutics, 463*(2), 127-136.

[162] Jiang, T., Carbone, E. J., Lo, K. W. H., & Laurencin, C. T. (2015). *Electrospinning of polymer nanofibers for tissue regeneration. Progress in Polymer Science, 46*, 1-24.

[163] Zhang, S., Huang, Y., Yang, X., Mei, F., Ma, Q., Chen, G., & Deng, X. (2009). Gelatin nanofibrous membrane fabricated by electrospinning of aqueous gelatin solution for guided tissue regeneration. *Journal of Biomedical Materials Research Part A, 90*(3), 671-679.

[164] Noritake, K., Kuroda, S., Myat, N. Y. A. N., Tabata, Y., & Kasugai, S. (2011). Development of a new barrier membrane for guided bone regeneration: an in vitro and in vivo study. *Journal of Oral Tissue Engineering, 9*(2), 53-63.

[165] Chou, J., Komuro, M., Hao, J., Kuroda, S., Hattori, Y., Ben-Nissan, B., & Otsuka, M. (2014). Bioresorbable zinc hydroxyapatite guided bone regeneration membrane for bone regeneration. *Clinical oral implants research*.

[166] Altman, G. H., Diaz, F., Jakuba, C., Calabro, T., Horan, R. L., Chen, J., & Kaplan, D. L. (2003). Silk-based biomaterials. *Biomaterials, 24*(3), 401-416.

[167] Santin, M., Motta, A., Freddi, G., & Cannas, M. (1999). In vitro evaluation of the inflammatory potential of the silk fibroin. *Journal of biomedical materials research, 46*(3), 382-389.

[168] Kim, K. H., Jeong, L., Park, H. N., Shin, S. Y., Park, W. H., Lee, S. C., ... & Ku, Y. (2005). Biological efficacy of silk fibroin nanofiber membranes for guided bone regeneration. *Journal of biotechnology, 120*(3), 327-339.

[169] Ha, Y. Y., Park, Y. W., Kweon, H., Jo, Y. Y., & Kim, S. G. (2014). Comparison of the physical properties and in vivo bioactivities of silkworm-cocoon-derived silk membrane, collagen membrane, and polytetrafluoroethylene membrane for guided bone regeneration. *Macromolecular Research, 22*(9), 1018-1023.

[170] Dupoirieux, L., Pourquier, D., Picot, M. C., & Neves, M. (2001). Comparative study of three different membranes for guided bone regeneration of rat cranial defects. *International journal of oral and maxillofacial surgery, 30*(1), 58-62.

[171] Ozmeric, N., Bal, B., Oygür, T., & Balos, K. (1999). The effect of a collagen membrane in regenerative therapy of two-wall intrabony defects in dogs. *Periodontal clinical investigations: official publication of the Northeastern Society of Periodontists*, *22*(1), 22-30.

[172] Gotfredsen, K., Nimb, L., & Hjørting-hansen, E. (1994). Immediate implant placement using a biodegradable barrier, polyhydroxybutyrate-hydroxyvalerate reinforced with polyglactin 910. An experimental study in dogs. *Clinical Oral Implants Research, 5*(2), 83-91.

[173] Kostopoulos, L., & Karring, T. (1994). Augmentation of the rat mandible using guided tissue regeneration. *Clinical oral implants research*, *5*(2), 75-82.

[174] Caffesse, R. G., Nasjleti, C. E., Morrison, E. C., & Sanchez, R. (1994). Guided tissue regeneration: comparison of bioabsorbable and non-bioabsorbable membranes. Histologic and histometric study in dogs. *Journal of periodontology, 65*(6), 583-591.

[175] Mundell, R. D., Mooney, M. P., Siegel, M. I., & Losken, A. (1993). Osseous guided tissue regeneration using a collagen barrier membrane. *Journal of oral and maxillofacial surgery, 51*(9), 1004-1012.

[176] Colangelo, P., Piattelli, A., Barrucci, S., Trisi, P., Formisano, G., & Caiazza, S. (1993). Bone regeneration guided by resorbable collagen membranes in rabbits: a pilot study. *Implant dentistry*, *2*(2), 101-106.

[177] Lundgren, D., Nyman, S., Mathisen, T., Isaksson, S., & Klinge, B. (1992). Guided bone regeneration of cranial defects, using biodegradable barriers: an experimental pilot study in the rabbit. *Journal of Cranio-Maxillofacial Surgery, 20*(6), 257-260.

[178] Oh, S. H., Park, I. K., Kim, J. M., & Lee, J. H. (2007). In vitro and in vivo characteristics of PCL scaffolds with pore size gradient fabricated by a centrifugation method. *Biomaterials, 28*(9), 1664-1671.

[179] Saito, E., Liao, E. E., Hu, W. W., Krebsbach, P. H., & Hollister, S. J. (2013). Effects of designed PLLA and 50: 50 PLGA scaffold architectures on bone formation in vivo. *Journal of tissue engineering and regenerative medicine, 7*(2), 99-111.

[180] Holland, T. A., & Mikos, A. G. (2005). Review: Biodegradable polymeric scaffolds. Improvements in bone tissue engineering through controlled drug delivery. *In Tissue Engineering I* (pp. 161-185). Springer Berlin Heidelberg.

[181] Shen, H., Hu, X., Yang, F., Bei, J., & Wang, S. (2009). The bioactivity of rhBMP-2 immobilized poly (lactide-co-glycolide) scaffolds. *Biomaterials, 30*(18), 3150-3157.

[182] Laurell, L., & Gottlow, J. (1998). Guided tissue regeneration update. *International dental journal, 48*(4), 386-398.

[183] Saiz-Arroyo, C., Wang, Y., Rodriguez-Perez, M. A., Alves, N. M., & Mano, J. F. (2007). In vitro monitoring of surface mechanical properties of poly (L-lactic acid) using microhardness. *Journal of applied polymer science, 105*(6), 3860-3864.

[184] Chen, Q., Zhu, C., & Thouas, G. A. (2012). Progress and challenges in biomaterials used for bone tissue engineering: bioactive glasses and elastomeric composites. *Progress in Biomaterials, 1*(1), 2.

[185] Tanase, C. E., & Spiridon, I. (2014). PLA/chitosan/keratin composites for biomedical applications. *Materials Science and Engineering: C, 40*, 242-247.

[186] Atala, A. (2002). Methods of tissue engineering. Gulf Professional Publishing.

[187] Matsusue, Y., Hanafusa, S., Yamamuro, T., Shikinami, Y., & Ikada, Y. (1995). Tissue Reaction of Bioabsorbable Ultra High Strength Poly (L-Lactide) Rod: A Long-Term Study in Rabbits. *Clinical orthopaedics and related research, 317*, 246-253.

[188] Meek, M. F., Jansen, K., Steendam, R., Oeveren, W., van Wachem, P. B., & van Luyn, M. J. (2004). In vitro degradation and biocompatibility of poly (DL-lactide-ϵ-caprolactone) nerve guides. *Journal of Biomedical Materials Research Part A, 68*(1), 43-51.

[189] Takata, T., Miyauchi, M., & Wang, H. L. (2001). Migration of osteoblastic cells on various guided bone regeneration membranes. *Clinical oral implants research, 12*(4), 332-338.

[190] Aurer, A., & JorgiE-Srdjak, K. (2005). Membranes for periodontal regeneration. *Acta Stomatol Croat, 39*(1), 95-112.

[191] Wu, L., & Ding, J. (2004). In vitro degradation of three-dimensional porous poly (D, L-lactide-co-glycolide) scaffolds for tissue engineering. *Biomaterials, 25*(27), 5821-5830.

[192] Göpferich, A. (1996). Mechanisms of polymer degradation and erosion. *Biomaterials, 17*(2), 103-114.

[193] Pontoriero, R., Wennström, J., & Lindhe, J. (1999). The use of barrier membranes and enamel matrix proteins in the treatment of angular bone defects. *A prospective controlled clinical study. Journal of Clinical Periodontology, 26*(12), 833-840.

[194] Donos, N., Kostopoulos, L., & Karring, T. (2002). Alveolar ridge augmentation using a resorbable copolymer membrane and autogenous bone grafts. *Clinical Oral Implants Research, 13*(2), 203-213.

[195] Jung, R. E., Kokovic, V., Jurisic, M., Yaman, D., Subramani, K., & Weber, F. E. (2011). Guided bone regeneration with a synthetic biodegradable membrane: a comparative study in dogs. *Clinical oral implants research, 22*(8), 802-807.

[196] Karfeld-Sulzer, L. S., Ghayor, C., Siegenthaler, B., Gjoksi, B., Pohjonen, T. H., & Weber, F. E. (2014). Comparative study of NMP-preloaded and dip-loaded membranes for guided bone regeneration of rabbit cranial defects. *Journal of tissue engineering and regenerative medicine.*

[197] Jamuna-Thevi, K., Saarani, N. N., Kadir, M. R. A., & Hermawan, H. (2014). Triple-layered PLGA/nanoapatite/lauric acid graded composite membrane for periodontal guided bone regeneration. *Materials Science and Engineering: C, 43*, 253-263.

[198] Santis, R., Gloria, A., Russo, T., D'Amora, U., D'Antò, V., Bollino, F., & Ambrosio, L. (2013). Advanced composites for hard-tissue engineering based on PCL/organic–inorganic hybrid fillers: From the design of 2D substrates to 3D rapid prototyped scaffolds. *Polymer Composites, 34*(9), 1413-1417.

[199] Domingos, M., Intranuovo, F., Russo, T., De Santis, R., Gloria, A., Ambrosio, L., & Bartolo, P. (2013). The first systematic analysis of 3D rapid prototyped poly (ε-caprolactone) scaffolds manufactured through BioCell printing: the effect of pore size and geometry on compressive mechanical behaviour and in vitro hMSC viability. *Biofabrication, 5*(4), 045004.

[200] De Santis, R., Russo, A., Gloria, A., D'Amora, U., Russo, T., Panseri, S., & Wilde, C. J. (2015). Towards the design of 3D fiber-deposited poly (caprolactone)/iron-doped hydroxyapatite nanocomposite magnetic scaffolds for bone regeneration. Journal of biomedical nanotechnology, 11(7), 1236-1246.

[201] Kim, Jong Young, Jun Jin Yoon, Eui Kyun Park, Dae Shick Kim, Shin-Yoon Kim, and Dong-Woo Cho. "Cell adhesion and proliferation evaluation of SFF-based biodegradable scaffolds fabricated using a multi-head deposition system." *Biofabrication 1, no. 1* (2009): 015002.

[202] Zhang, E., Zhu, C., Yang, J., Sun, H., Zhang, X., Li, S., ... & Yao, F. (2016). Electrospun PDLLA/PLGA composite membranes for potential application in guided tissue regeneration. *Materials Science and Engineering: C, 58*, 278-285.

[203] Allori, A. C., Sailon, A. M., & Warren, S. M. (2008). Biological basis of bone formation, remodeling, and repair—part I: biochemical signaling molecules. *Tissue Engineering Part B: Reviews, 14*(3), 259-273.

[204] Fielding, G., & Bose, S. (2013). SiO 2 and ZnO dopants in three-dimensionally printed tricalcium phosphate bone tissue engineering scaffolds enhance osteogenesis and angiogenesis in vivo. *Acta biomaterialia*, *9*(11), 9137-9148.

[205] Pawlik, J., Widziołek, M., Cholewa-Kowalska, K., Łączka, M., & Osyczka, A. M. (2014). New sol-gel bioactive glass and titania composites with enhanced physico-chemical and biological properties. *Journal of Biomedical Materials Research Part A*, *102*(7), 2383-2394.

[206] Tiainen, H., Wohlfahrt, J. C., Verket, A., Lyngstadaas, S. P., & Haugen, H. J. (2012). Bone formation in TiO 2 bone scaffolds in extraction sockets of minipigs. *Acta biomaterialia*, *8*(6), 2384-2391.

[207] Vallet-Regí, M., & Ruiz-Hernández, E. (2011). Bioceramics: from bone regeneration to cancer nanomedicine. *Advanced Materials*, *23*(44), 5177-5218.

[208] Damien, C. J., & Parsons, J. R. (1991). Bone graft and bone graft substitutes: a review of current technology and applications. *Journal of Applied Biomaterials*, *2*(3), 187-208.

[209] Barrère, F., van Blitterswijk, C. A., & de Groot, K. (2006). Bone regeneration: molecular and cellular interactions with calcium phosphate ceramics. *International Journal of Nanomedicine*, *1*(3), 317.

[210] LeGeros, R. Z. (2008). Calcium phosphate-based osteoinductive materials. *Chemical reviews*, *108*(11), 4742-4753.

[211] Müller, P., Bulnheim, U., Diener, A., Lüthen, F., Teller, M., Klinkenberg, E. D. & Rychly, J. (2008). Calcium phosphate surfaces promote osteogenic differentiation of mesenchymal stem cells. *Journal of cellular and molecular medicine*, *12*(1), 281-291.

[212] Yuan, H., Fernandes, H., Habibovic, P., de Boer, J., Barradas, A. M., de Ruiter, A., & de Bruijn, J. D. (2010). Osteoinductive ceramics as a synthetic alternative to autologous bone grafting. *Proceedings of the National Academy of Sciences*, *107*(31), 13614-13619.

[213] Jose, M. V., Thomas, V., Johnson, K. T., Dean, D. R., & Nyairo, E. (2009). Aligned PLGA/HA nanofibrous nanocomposite scaffolds for bone tissue engineering. *Acta biomaterialia*, *5*(1), 305-315.

[214] Jose, M. V., Thomas, V., Xu, Y., Bellis, S., Nyairo, E., & Dean, D. (2010). Aligned bioactive multi-component nanofibrous nanocomposite scaffolds for bone tissue engineering. *Macromolecular bioscience*, *10*(4), 433-444.

[215] Prabhakaran, M. P., Venugopal, J., & Ramakrishna, S. (2009). Electrospun nanostructured scaffolds for bone tissue engineering. *Acta Biomaterialia*, *5*(8), 2884-2893.

[216] Wutticharoenmongkol, P., Sanchavanakit, N., Pavasant, P., & Supaphol, P. (2006). Preparation and characterization of novel bone scaffolds based on electrospun polycaprolactone fibers filled with nanoparticles. *Macromolecular bioscience*, *6*(1), 70-77.

[217] Ngiam, M., Liao, S., Patil, A. J., Cheng, Z., Chan, C. K., & Ramakrishna, S. (2009). The fabrication of nano-hydroxyapatite on PLGA and PLGA/collagen nanofibrous composite scaffolds and their effects in osteoblastic behavior for bone tissue engineering. *Bone*, *45*(1), 4-16.

[218] Venugopal, J., Low, S., Choon, A. T., Kumar, T. S., & Ramakrishna, S. (2008). Mineralization of osteoblasts with electrospun collagen/hydroxyapatite nanofibers. *Journal of Materials Science: Materials in Medicine*, *19*(5), 2039-2046.

[219] Dvir, T., Timko, B. P., Kohane, D. S., & Langer, R. (2011). Nanotechnological strategies for engineering complex tissues. *Nature nanotechnology*, *6*(1), 13-22.

[220] Fujita, N., Matsushita, T., Ishida, K., Sasaki, K., Kubo, S., Matsumoto, T., & Kuroda, R. (2012). An analysis of bone regeneration at a segmental bone defect by controlled release of bone morphogenetic protein 2 from a biodegradable sponge composed of gelatin and β-tricalcium phosphate. *Journal of tissue engineering and regenerative medicine*, *6*(4), 291-298.

[221] Chen, J. P., Tsai, M. J., & Liao, H. T. (2013). Incorporation of biphasic calcium phosphate microparticles in injectable thermoresponsive hydrogel modulates bone cell proliferation and differentiation. *Colloids and Surfaces B: Biointerfaces*, *110*, 120-129.

[222] Hirota, M., Matsui, Y., Mizuki, N., Kishi, T., Watanuki, K., Ozawa, T., & Iwai, T. (2009). Combination with allogenic bone reduces early absorption of. BETA. -tricalcium phosphate (. BETA. -TCP) and enhances the role as a bone regeneration scaffold. Experimental animal study in rat mandibular bone defects. *Dental materials journal*, *28*(2), 153-161.

[223] Jansen, J. A., De Ruijter, J. E., Janssen, P. T. M., & Paquay, Y. G. C. J. (1995). Histological evaluation of a biodegradable polyactive®/hydroxyapatite membrane. *Biomaterials*, *16*(11), 819-827.

[224] Li, C., Vepari, C., Jin, H. J., Kim, H. J., & Kaplan, D. L. (2006). Electrospun silk-BMP-2 scaffolds for bone tissue engineering. *Biomaterials*, *27*(16), 3115-3124.

[225] Thomas, V., Jagani, S., Johnson, K., Jose, M. V., Dean, D. R., Vohra, Y. K., & Nyairo, E. (2006). Electrospun bioactive nanocomposite scaffolds of polycaprolactone and nanohydroxyapatite for bone tissue engineering. *Journal of nanoscience and nanotechnology*, *6*(2), 487-493.

[226] Vaquette, C., & Cooper-White, J. (2013). A simple method for fabricating 3-D multilayered composite scaffolds. *Acta biomaterialia*, *9*(1), 4599-4608.

[227] Xu, H., Cui, W., & Chang, J. (2013). Fabrication of patterned PDLLA/PCL composite scaffold by electrospinning. *Journal of Applied Polymer Science*, *127*(3), 1550-1554.

[228] Kim, S., Nimni, M. E., Yang, Z., & Han, B. (2005). Chitosan/gelatin–based films crosslinked by proanthocyanidin. *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, *75*(2), 442-450.

[229] Hunter, K. T., & Ma, T. (2013). In vitro evaluation of hydroxyapatite–chitosan–gelatin composite membrane in guided tissue regeneration. *Journal of Biomedical Materials Research Part A*, *101*(4), 1016-1025.

[230] Shi, R., Xue, J., He, M., Chen, D., Zhang, L., & Tian, W. (2014). Structure, physical properties, biocompatibility and in vitro/vivo degradation behavior of anti-infective polycaprolactone-based electrospun membranes for guided tissue/bone regeneration. *Polymer Degradation and Stability*, *109*, 293-306.

[231] Xue, J., He, M., Liang, Y., Crawford, A., Coates, P., Chen, D., ... & Zhang, L. (2014). Fabrication and evaluation of electrospun PCL–gelatin micro-/nanofiber membranes for anti-infective GTR implants. *Journal of Materials Chemistry B*, *2*(39), 6867-6877.

[232] Ji, W., Yang, F., Ma, J., Bouma, M. J., Boerman, O. C., Chen, Z., & Jansen, J. A. (2013). Incorporation of stromal cell-derived factor-1α in PCL/gelatin electrospun membranes for guided bone regeneration. *Biomaterials*, *34*(3), 735-745.

[233] Shi, R., Xue, J., Wang, H., Wang, R., Gong, M., Chen, D., & Tian, W. (2015). Fabrication and evaluation of a homogeneous electrospun PCL–gelatin hybrid membrane as an anti-adhesion barrier for craniectomy. *Journal of Materials Chemistry B*, *3*(19), 4063-4073.

[234] Xue, J., He, M., Liu, H., Niu, Y., Crawford, A., Coates, P. D., & Zhang, L. (2014). Drug loaded homogeneous electrospun PCL/gelatin hybrid nanofiber structures for anti-infective tissue regeneration membranes. *Biomaterials*, *35*(34), 9395-9405.

[235] Chen, S., Hao, Y., Cui, W., Chang, J., & Zhou, Y. (2013). Biodegradable electrospun PLLA/chitosan membrane as guided tissue regeneration membrane for treating periodontitis. *Journal of Materials Science*, *48*(19), 6567-6577.

[236] Jiang, W., Li, L., Zhang, D., Huang, S., Jing, Z., Wu, Y., & Zhou, S. (2015). Incorporation of aligned pcl–peg nanofibers into porous chitosan scaffolds improved the orientation of collagen fibers in regenerated periodontium. *Acta biomaterialia*, *25*, 240-252.

[237] Campoccia, D., Montanaro, L., & Arciola, C. R. (2013). A review of the clinical implications of anti-infective biomaterials and infection-resistant surfaces. *Biomaterials*, *34*(33), 8018-8029.

[238] Misra, S. K., Ansari, T. I., Valappil, S. P., Mohn, D., Philip, S. E., Stark, W. J., ... & Boccaccini, A. R. (2010). Poly (3-hydroxybutyrate) multifunctional composite scaffolds for tissue engineering applications. *Biomaterials*, *31*(10), 2806-2815.

[239] Liu, Y., Lim, J., & Teoh, S. H. (2013). Review: development of clinically relevant scaffolds for vascularised bone tissue engineering. *Biotechnology advances*, *31*(5), 688-705.

[240] Yang, K., Lee, S. H., & Oh, J. M. (1999). Effects of viscosity ratio and compatibilizers on the morphology and mechanical properties of polycarbonate/acrylonitrile-butadiene-styrene blends. *Polymer Engineering & Science*, *39*(9), 1667-1677.

[241] Helfand, E., & Sapse, A. M. (1975). Theory of unsymmetric polymer–polymer interfaces. *The Journal of Chemical Physics*, *62*(4), 1327-1331.

[242] Lindsey, C. R., Paul, D. R., & Barlow, J. W. (1981). Mechanical properties of HDPE–PS–SEBS blends. *Journal of Applied Polymer Science*, *26*(1), 1-8.

[243] Datta, S., & Lohse, D. J. (1996). Polymeric comptabilizers.

[244] Tao, Y., Kim, J., & Torkelson, J. M. (2006). Achievement of quasi-nanostructured polymer blends by solid-state shear pulverization and compatibilization by gradient copolymer addition. *Polymer*, *47*(19), 6773-6781.

[245] Creton, C., Kramer, E. J., & Hadziioannou, G. (1992). Craze fibril extension ratio measurements in glassy block copolymers. *Colloid & Polymer Science*, *270*(5), 399-404.

[246] Utracki, L. A. (2002). Compatibilization of polymer blends. *The Canadian Journal of Chemical Engineering*, *80*(6), 1008-1016.

[247] Macosko, C. W., Guegan, P., Khandpur, A. K., Nakayama, A., Marechal, P., & Inoue, T. (1996). Compatibilizers for melt blending: Premade block copolymers. *Macromolecules*, *29*(17), 5590-5598.

[248] Sandoval, R. W., Williams, D. E., Kim, J., Roth, C. B., & Torkelson, J. M. (2008). Critical micelle concentrations of block and gradient copolymers in homopolymer: effects of sequence distribution, composition, and molecular weight. *Journal of Polymer Science Part B: Polymer Physics*, *46*(24), 2672-2682.

[249] Ide, F., & Hasegawa, A. (1974). Studies on polymer blend of nylon 6 and polypropylene or nylon 6 and polystyrene using the reaction of polymer. *Journal of applied polymer science*, *18*(4), 963-974.

[250] Allaf, R. M., & Rivero, I. V. (2011). Fabrication and characterization of interconnected porous biodegradable poly (ε-caprolactone) load bearing scaffolds. *Journal of Materials Science: Materials in Medicine*, *22*(8), 1843-1853.

[251] Koning, C., Van Duin, M., Pagnoulle, C., & Jerome, R. (1998). Strategies for compatibilization of polymer blends. *Progress in Polymer Science*, *23*(4), 707-757.

[252] Allaf, R. M., Rivero, I. V., Abidi, N., & Ivanov, I. N. (2013). Porous poly (ε-caprolactone) scaffolds for load-bearing tissue regeneration: Solventless fabrication and characterization. *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, *101*(6), 1050-1060.

[253] Zhu, Y. G., Li, Z. Q., Gu, J. J., Zhang, D., & Tanimoto, T. (2006). Polyaniline/iron nanocomposites prepared by cryomilling. *Journal of Polymer Science Part B: Polymer Physics*, *44*(21), 3157-3164.

[254] Chen, C. J., & Liao, S. L. (2003). Zinc toxicity on neonatal cortical neurons: involvement of glutathione chelation. *Journal of neurochemistry*, *85*(2), 443-453.

[255] Jones, J., Heiselman, D., Dougherty, J., & Eddy, A. (1986). Cathartic-induced magnesium toxicity during overdose management. *Annals of emergency medicine*, *15*(10), 1214-1218.

[256] Hou, Q., Shi, Z. C., Fan, R. H., & Ju, L. C. (2012). Cryomilling and characterization of metal/ceramic powders. In *Key Engineering Materials* (Vol. 512, pp. 127-131). Trans Tech Publications.

[257] Cao, H., & Kuboyama, N. (2010). A biodegradable porous composite scaffold of PGA/β-TCP for bone tissue engineering. *Bone*, *46*(2), 386-395.

[258] Zein, I., Hutmacher, D. W., Tan, K. C., & Teoh, S. H. (2002). Fused deposition modeling of novel scaffold architectures for tissue engineering applications. *Biomaterials*, *23*(4), 1169-1185.

[259] Coroller, G., Lefeuvre, A., Le Duigou, A., Bourmaud, A., Ausias, G., Gaudry, T., & Baley, C. (2013). Effect of flax fibres individualisation on tensile failure of flax/epoxy unidirectional composite. *Composites Part A: Applied Science and Manufacturing*, *51*, 62-70.

[260] Bose, S., & Bogner, R. H. (2007). Solventless pharmaceutical coating processes: a review. *Pharmaceutical development and technology*, *12*(2), 115-131.

[261] Chua, C. K., Leong, K. F., & An, J. (2014). Introduction to rapid prototyping of biomaterials. *Rapid prototyping of biomaterials. Woodhead Publishing, Philadelphia*, 1-15.

[262] Jonnalagadda, J. B., & Rivero, I. V. (2014). Effect of cryomilling times on the resultant properties of porous biodegradable poly (e-caprolactone)/poly (glycolic acid) scaffolds for articular cartilage tissue engineering. *Journal of the mechanical behavior of biomedical materials*, *40*, 33-41.

[263] Suryanarayana, C. (2001). Mechanical alloying and milling. *Progress in materials science*, *46*(1), 1-184.

[264] Raffi, M., Hussain, F., Bhatti, T. M., Akhter, J. I., Hameed, A., & Hasan, M. M. (2008). Antibacterial characterization of silver nanoparticles against E. coli ATCC-15224. *Journal of Materials Science and Technology*, *24*(2), 192-196.

[265] Barnes, C. P., Sell, S. A., Boland, E. D., Simpson, D. G., & Bowlin, G. L. (2007). Nanofiber technology: designing the next generation of tissue engineering scaffolds. *Advanced drug delivery reviews*, *59*(14), 1413-1433.

[266] Li, W. J., Mauck, R. L., & Tuan, R. S. (2005). Electrospun nanofibrous scaffolds: production, characterization, and applications for tissue engineering and drug delivery. *Journal of Biomedical Nanotechnology*, *1*(3), 259-275.

[267] Pham, Q. P., Sharma, U., & Mikos, A. G. (2006). Electrospinning of polymeric nanofibers for tissue engineering applications: a review. *Tissue engineering*, *12*(5), 1197-1211.

[268] Kumbar, S. G., Nair, L. S., Bhattacharyya, S., & Laurencin, C. T. (2006). Polymeric nanofibers as novel carriers for the delivery of therapeutic molecules. *Journal of nanoscience and nanotechnology*, *6*(9-1), 2591-2607.

[269] Toh, Y. C., Ng, S., Khong, Y. M., Zhang, X., Zhu, Y., Lin, P. C., & Yu, H. (2006). Cellular responses to a nanofibrous environment. *Nano Today*, *1*(3), 34-43.

[270] Matthews, J. A., Wnek, G. E., Simpson, D. G., & Bowlin, G. L. (2002). Electrospinning of collagen nanofibers. *Biomacromolecules*, *3*(2), 232-238.

[271] Zhang, S. (2003). Fabrication of novel biomaterials through molecular self-assembly. *Nature biotechnology*, *21*(10), 1171-1178.

[272] Yoo, H. S., Kim, T. G., & Park, T. G. (2009). Surface-functionalized electrospun nanofibers for tissue engineering and drug delivery. *Advanced drug delivery reviews*, *61*(12), 1033-1042.

[273] Chew, S. Y., Hufnagel, T. C., Lim, C. T., & Leong, K. W. (2006). Mechanical properties of single electrospun drug-encapsulated nanofibres. *Nanotechnology*, *17*(15), 3880.

[274] Van de Witte, P., Dijkstra, P. J., Van den Berg, J. W. A., & Feijen, J. (1996). Phase separation processes in polymer solutions in relation to membrane formation. *Journal of Membrane Science*, *117*(1-2), 1-31.

[275] Mikos, A. G., & Temenoff, J. S. (2000). Formation of highly porous biodegradable scaffolds for tissue engineering. *Electronic Journal of Biotechnology*, *3*(2), 23-24.

[276] Zhao, J., Han, W., Chen, H., Tu, M., Zeng, R., Shi, Y., & Zhou, C. (2011). Preparation, structure and crystallinity of chitosan nano-fibers by a solid–liquid phase separation technique. *Carbohydrate polymers*, *83*(4), 1541-1546.

[277] Ma, P. X., & Zhang, R. (1999). Synthetic nano-scale fibrous extracellular matrix.

[278] Li, X. T., Zhang, Y., & Chen, G. Q. (2008). Nanofibrous polyhydroxyalkanoate matrices as cell growth supporting materials. *Biomaterials*, *29*(27), 3720-3728.

[279] Rexeisen, E. L., Fan, W., Pangburn, T. O., Taribagil, R. R., Bates, F. S., Lodge, T. P., & Kokkoli, E. (2009). Self-assembly of fibronectin mimetic peptide-amphiphile nanofibers. *Langmuir*, *26*(3), 1953-1959.

[280] Cui, H., Webber, M. J., & Stupp, S. I. (2010). Self-assembly of peptide amphiphiles: From molecules to nanostructures to biomaterials. *Peptide Science*, *94*(1), 1-18.

[281] Paramonov, S. E., Jun, H. W., & Hartgerink, J. D. (2006). Self-assembly of peptide− amphiphile nanofibers: the roles of hydrogen bonding and amphiphilic packing. *Journal of the American Chemical Society*, *128*(22), 7291-7298.

[282] Xu, C. Y., Inai, R., Kotaki, M., & Ramakrishna, S. (2004). Aligned biodegradable nanofibrous structure: a potential scaffold for blood vessel engineering. *Biomaterials*, *25*(5), 877-886.

[283] Yin, Z., Chen, X., Chen, J. L., Shen, W. L., Nguyen, T. M. H., Gao, L., & Ouyang, H. W. (2010). The regulation of tendon stem cell differentiation by the alignment of nanofibers. *Biomaterials*, *31*(8), 2163-2175.

[284] Dahlin, R. L., Kasper, F. K., & Mikos, A. G. (2011). Polymeric nanofibers in tissue engineering. *Tissue Engineering Part B: Reviews*, *17*(5), 349-364.

[285] Zhang, Y. Z., Wang, X., Feng, Y., Li, J., Lim, C. T., & Ramakrishna, S. (2006). Coaxial electrospinning of (fluorescein isothiocyanate-conjugated bovine serum albumin)-encapsulated poly (ε-caprolactone) nanofibers for sustained release. *Biomacromolecules*, *7*(4), 1049-1057.

[286] Pham, Q. P., Sharma, U., & Mikos, A. G. (2006). Electrospun poly (ε-caprolactone) microfiber and multilayer nanofiber/microfiber scaffolds: characterization of scaffolds and measurement of cellular infiltration. *Biomacromolecules*, *7*(10), 2796-2805.

[287] Li, W. J., Tuli, R., Okafor, C., Derfoul, A., Danielson, K. G., Hall, D. J., & Tuan, R. S. (2005). A three-dimensional nanofibrous scaffold for cartilage tissue engineering using human mesenchymal stem cells. *Biomaterials*, *26*(6), 599-609.

[288] Yang, F., Murugan, R., Wang, S., & Ramakrishna, S. (2005). Electrospinning of nano/micro scale poly (L-lactic acid) aligned fibers and their potential in neural tissue engineering. *Biomaterials*, *26*(15), 2603-2610.

[289] Lee, C. H., Shin, H. J., Cho, I. H., Kang, Y. M., Kim, I. A., Park, K. D., & Shin, J. W. (2005). Nanofiber alignment and direction of mechanical strain affect the ECM production of human ACL fibroblast. *Biomaterials*, *26*(11), 1261-1270.

[290] Choi, J. S., Leong, K. W., & Yoo, H. S. (2008). In vivo wound healing of diabetic ulcers using electrospun nanofibers immobilized with human epidermal growth factor (EGF). *Biomaterials*, *29*(5), 587-596.

[291] Zhou, Y., Yang, D., Chen, X., Xu, Q., Lu, F., & Nie, J. (2007). Electrospun water-soluble carboxyethyl chitosan/poly (vinyl alcohol) nanofibrous membrane as potential wound dressing for skin regeneration. *Biomacromolecules*, *9*(1), 349-354.

[292] Sui, G., Yang, X., Mei, F., Hu, X., Chen, G., Deng, X., & Ryu, S. (2007). Poly-L-lactic acid/hydroxyapatite hybrid membrane for bone tissue regeneration. *Journal of Biomedical Materials Research Part A*, *82*(2), 445-454.

[293] Deng, X. L., Sui, G., Zhao, M. L., Chen, G. Q., & Yang, X. P. (2007). Poly (L-lactic acid)/hydroxyapatite hybrid nanofibrous scaffolds prepared by electrospinning. *Journal of Biomaterials Science, Polymer Edition*, *18*(1), 117-130.

[294] Kim, H. W., Lee, H. H., & Knowles, J. C. (2006). Electrospinning biomedical nanocomposite fibers of hydroxyapatite/poly (lactic acid) for bone regeneration. *Journal of biomedical materials research part A*, *79*(3), 643-649.

[295] Mei, F., Zhong, J., Yang, X., Ouyang, X., Zhang, S., Hu, X., & Deng, X. (2007). Improved biological characteristics of poly (L-lactic acid) electrospun membrane by incorporation of Multiwalled carbon nanotubes/hydroxyapatite nanoparticles. *Biomacromolecules*, *8*(12), 3729-3735.

[296] Schneider, O. D., Loher, S., Brunner, T. J., Uebersax, L., Simonet, M., Grass, R. N., & Stark, W. J. (2008). Cotton wool-like nanocomposite biomaterials prepared by electrospinning: In vitro bioactivity and osteogenic differentiation of human mesenchymal stem cells. *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, *84*(2), 350-362.

[297] Chen, F., Li, X., Mo, X., He, C., Wang, H., & Ikada, Y. (2008). Electrospun chitosan-P (LLA-CL) nanofibers for biomimetic extracellular matrix. *Journal of Biomaterials Science, Polymer Edition*, *19*(5), 677-691.

[298] Yang, D., Jin, Y., Ma, G., Chen, X., Lu, F., & Nie, J. (2008). Fabrication and characterization of chitosan/PVA with hydroxyapatite biocomposite nanoscaffolds. *Journal of applied polymer science*, *110*(6), 3328-3335.

[299] Zhang, Y., Venugopal, J. R., El-Turki, A., Ramakrishna, S., Su, B., & Lim, C. T. (2008). Electrospun biomimetic nanocomposite nanofibers of hydroxyapatite/chitosan for bone tissue engineering. *Biomaterials*, *29*(32), 4314-4322.

[300] Badami, A. S., Kreke, M. R., Thompson, M. S., Riffle, J. S., & Goldstein, A. S. (2006). Effect of fiber diameter on spreading, proliferation, and differentiation of osteoblastic cells on electrospun poly (lactic acid) substrates. *Biomaterials*, *27*(4), 596-606.

[301] Hu, H. T., Lee, S. Y., Chen, C. C., Yang, Y. C., & Yang, J. C. (2013). Processing and properties of hydrophilic electrospun polylactic acid/beta-tricalcium phosphate membrane for dental applications. *Polymer Engineering & Science*, *53*(4), 833-842.

[302] McCullen, S. D., Zhu, Y., Bernacki, S. H., Narayan, R. J., Pourdeyhimi, B., Gorga, R. E., & Loboa, E. G. (2009). Electrospun composite poly (L-lactic acid)/tricalcium phosphate scaffolds induce proliferation and osteogenic differentiation of human adipose-derived stem cells. *Biomedical materials*, *4*(3), 035002.

[303] Zhao, X. F., Li, X. D., Kang, Y. Q., & Yuan, Q. (2011). Improved biocompatibility of novel poly (L-lactic acid)/β-tricalcium phosphate scaffolds prepared by an organic solvent-free method. *International journal of nanomedicine*, *6*, 1385-1390.

[304] Kang, Y., Yin, G., Yuan, Q., Yao, Y., Huang, Z., Liao, X., & Wang, H. (2008). Preparation of poly (L-lactic acid)/β-tricalcium phosphate scaffold for bone tissue engineering without organic solvent. *Materials Letters*, *62*(12), 2029-2032.

[305] Pakravan, M., Heuzey, M. C., & Ajji, A. (2011). A fundamental study of chitosan/PEO electrospinning. *Polymer*, *52*(21), 4813-4824.

[306] Xu, J., Zhang, J., Gao, W., Liang, H., Wang, H., & Li, J. (2009). Preparation of chitosan/PLA blend micro/nanofibers by electrospinning. *Materials Letters*, *63*(8), 658-66.

[307] Razali, N. M., & Wah, Y. B. (2011). Power comparisons of shapiro-wilk, kolmogorov-smirnov, lilliefors and anderson-darling tests. *Journal of statistical modeling and analytics*, *2*(1), 21-33.

## REFERENCES FOR FIGURES

[1] WHY DO I NEED IT? Retrieved from https://www.stellardentalclt.com/scaling-and-root-planing.

[2] 1- Intro to Perio, University of Maryland, Baltimore. Retrieved from https://www.studyblue.com/notes/note/n/1-intro-to-perio/deck/13723414

[3] Surgical Orthodontics, Marcos Diaz, DDS. Retrieved from http://facialsurg.cc/Biologic-and-new-arising-technology.php

[4] Henry, M. F. (2010). Solid-State Compatibilization of Immiscible Polymer Blends: Cryogenic Milling and Solid-State Shear Pulverization (Unpublished master's thesis). Bucknell University, Pennsylvania.

$$\mu l/ml\ \mu l/ml, \mu l/ml\ \mu l/ml\ \mu\ \mu\ \mu W_t = \frac{(\sum_{i=1}^{n} a_i x_{(i)})^2}{\sum_{i=1}^{n}(x_i - \bar{x})^2} x_{(i)} \bar{x}\, a_t a_i, a_i, \dots\dots., a_i) \frac{m^T V^{-1}}{\sqrt{\quad}} (m_1, m_2, \dots\dots\dots, m_n)^T m_t q_s \frac{Y_A - Y_B}{SE} Y_A Y_B\ \mu l /ml)$$

## APPENDIX STATISTICAL ANALYSIS

In this study, statistical analyses (Two-way ANOVA with post hoc Tukey's test) were performed to assist in evaluating the credibility of the results obtained from the *In vitro* studies. The analyses were carried out with PRISM ver. 7.0 software. For all the tests, values of $p<0.05$ were taken to indicate statistical significance (95% confidence level). This section intends to summarize all the details of the statistical analyses that were performed.

### 1. Analyses for CTB® Cell Viability Assay with RAW 264.7 Murine Macrophages

The cytotoxicity test was conducted with three samples in each treatment group, they were all tested under the same condition within each group, so the measured fluorescence intensity values were expected to have the same distribution. The samples were prepared individually and tested in individual wells. So, it was assumed that the observed values of fluorescence intensity under each treatment were independent and identically distributed random variables. This experiment had two independent variables, namely, material composition (A0, A1, A2, and A3) and powder concentration (3 µl/ml, 10 µl/ml, 30 µl/ml and 50 µl/ml). The fluorescence intensity values obtained from the micro-plate reader was the only dependent variable.

Three major assumptions needed to be satisfied before the use of a two-way ANOVA test. As previously mentioned, the independence of cases assumption was met by preparing and testing samples individually. The homogeneity of variance assumption was also checked before proceeding with the parametric test. The Shapiro-Wilk W test was used to test for normality of the

data as it has been shown to have a higher power in case of small sample sizes in comparison to other normality tests [307]. The corresponding null hypothesis was that the fluorescence intensity values were normally distributed. Fig. 1 and Fig. 2 show sample results of the Shapiro-Wilk W Test for the cytotoxicity tests performed after 24 and 48 h from cell seeding (A1-3 μl/ml combination).

| Shapiro-Wilk normality test | |
|---|---|
| W | 0.8339 |
| P value | 0.1983 |
| Passed normality test (alpha=0.05)? | Yes |

**Fig.1.** Normality test results for A1-3 μl/ml (24 h)

| Shapiro-Wilk normality test | |
|---|---|
| W | 0.9995 |
| P value | 0.9552 |
| Passed normality test (alpha=0.05)? | Yes |

**Fig. 2.** Normality test results for A1-3 μl/ml (48 h)

In the Shapiro-Wilk W Test, the test statistic is calculated using the following equation,

$$W_t = \frac{(\sum_{i=1}^{n} a_i x_{(i)})^2}{\sum_{i=1}^{n} (x_i - \bar{x})^2} \quad \text{(A.1)}$$

In equation A.1, $i^{\text{th}}$ ordered statistic in the entire data set is given by $x_{(i)}$, $\bar{x}$ is the mean of all statistics and $a_i$ is given by equation A.2 as described below,

$$(a_i, a_i, \ldots\ldots, a_i) = \frac{m^T V^{-1}}{\sqrt{m^T V^{-1} V^{-1} m}} \quad \text{(A.2)}$$

In equation A.2, $m$ is defined as A.3,

$$m = (m_1, m_2, \ldots\ldots\ldots, m_n)^T \quad \text{(A.3)}$$

In A.3, $m_i$ are the expected values of the order statistics of independent and identically distributed random variables sampled from the standard normal distribution and in A.2, V is the covariance matrix of those order statistics. The value of W lies between zero and one. Small values of W lead to the rejection of normality whereas a value closer to one indicates normality of the data.

When the analysis was conducted, p values less than 0.05 were observed for all the treatment combinations, and hence, it was concluded that fluorescence intensity values were normally

distributed. Moreover, it also meant that the two-way ANOVA with post hoc Tukey's test could be used to statistically evaluate the difference between the means. In this study, the cells grown in the standard medium were used as controls.

The result of the two-way ANOVA test conducted for the samples cultured for 24 h is shown below in Fig. 3. The value of p for material composition was 0.0001 which was less than the designated Type I error rate of 0.005. Thus, it was concluded that the material composition had a considerable influence on the survival of the macrophages. However, the value of p for powder concentration was 0.0580. Hence, it was concluded that the concentration of the powder did not have a statistically significant influence on the survival of the macrophages. It was also seen that the independent variables did not have a statistically significant interaction effect on the dependent variable. However, in the case of statistical significance, the Tukey's test was performed to identify the groups that had a significant difference between them.

| | | | | | |
|---|---|---|---|---|---|
| Table Analyzed | RAW24 GIUSTO | | | | |
| | | | | | |
| Two-way ANOVA | Ordinary | | | | |
| Alpha | 0.05 | | | | |
| | | | | | |
| Source of Variation | % of total variation | P value | P value summary | Significant? | |
| Interaction | 4.7 | 0.6413 | ns | No | |
| POWDER CONCENTRATION | 3.939 | 0.0580 | ns | No | |
| MATERIAL COMPOSITION | 71.95 | <0.0001 | **** | Yes | |
| | | | | | |
| ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
| Interaction | 1504328253 | 12 | 125360688 | F (12, 40) = 0.8072 | P=0.6413 |
| POWDER CONCENTRATION | 1260843971 | 3 | 420281324 | F (3, 40) = 2.706 | P=0.0580 |
| MATERIAL COMPOSITION | 23028442281 | 4 | 5757110570 | F (4, 40) = 37.07 | P<0.0001 |
| Residual | 6211990739 | 40 | 155299768 | | |
| | | | | | |
| Number of missing values | 0 | | | | |

**Fig. 3.** Two-way ANOVA results for cytotoxicity test with macrophages (24 h)

The Tukey's test conducted for the samples cultured for 24 h revealed that the material compositions were statistically different from the control group but otherwise, A0, A1, A2 and A3 did not differ significantly from each other. Fig. 4 shows the results of the Tukey's test. The Tukey's test is calculated based on the equation A.4,

$$q_s = \frac{Y_A - Y_B}{SE} \tag{A.4}$$

where $Y_A$ the larger of the two means being compared is, $Y_B$ is the smaller of the two means being compared, and SE is the standard error of the data in question.

| Tukey's multiple comparisons test | Mean Diff. | 95.00% CI of diff. | Significant? | Summary | Adjusted P Value |
|---|---|---|---|---|---|
| | | | | | |
| 3ml | | | | | |
| A0 vs. A1 | 2798 | -26263 to 31859 | No | ns | 0.9987 |
| A0 vs. A2 | 5241 | -23820 to 34302 | No | ns | 0.9854 |
| A0 vs. A3 | -6024 | -35085 to 23037 | No | ns | 0.9755 |
| A0 vs. Controls | -55708 | -84770 to -26647 | Yes | **** | <0.0001 |
| A1 vs. A2 | 2443 | -26618 to 31504 | No | ns | 0.9992 |
| A1 vs. A3 | -8822 | -37883 to 20239 | No | ns | 0.9072 |
| A1 vs. Controls | -58507 | -87568 to -29446 | Yes | **** | <0.0001 |
| A2 vs. A3 | -11265 | -40326 to 17796 | No | ns | 0.8018 |
| A2 vs. Controls | -60950 | -90011 to -31888 | Yes | **** | <0.0001 |
| A3 vs. Controls | -49684 | -78745 to -20623 | Yes | *** | 0.0002 |
| | | | | | |
| 10ml | | | | | |
| A0 vs. A1 | 5465 | -23596 to 34526 | No | ns | 0.9829 |
| A0 vs. A2 | -16428 | -45490 to 12633 | No | ns | 0.4971 |
| A0 vs. A3 | -21700 | -50761 to 7361 | No | ns | 0.2267 |
| A0 vs. Controls | -50887 | -79948 to -21826 | Yes | *** | 0.0001 |
| A1 vs. A2 | -21894 | -50955 to 7167 | No | ns | 0.2191 |
| A1 vs. A3 | -27165 | -56226 to 1896 | No | ns | 0.0768 |
| A1 vs. Controls | -56352 | -85413 to -27291 | Yes | **** | <0.0001 |
| A2 vs. A3 | -5272 | -34333 to 23789 | No | ns | 0.9850 |
| A2 vs. Controls | -34459 | -63520 to -5398 | Yes | * | 0.0131 |
| A3 vs. Controls | -29187 | -58248 to -126 | Yes | * | 0.0486 |
| 30ml | | | | | |
| A0 vs. A1 | -9007 | -38068 to 20054 | No | ns | 0.9007 |
| A0 vs. A2 | -14400 | -43461 to 14661 | No | ns | 0.6217 |
| A0 vs. A3 | -20796 | -49857 to 8265 | No | ns | 0.2645 |
| A0 vs. Controls | -56286 | -85347 to -27225 | Yes | **** | <0.0001 |
| A1 vs. A2 | -5393 | -34454 to 23668 | No | ns | 0.9837 |
| A1 vs. A3 | -11789 | -40850 to 17272 | No | ns | 0.7744 |
| A1 vs. Controls | -47279 | -76340 to -18218 | Yes | *** | 0.0003 |
| A2 vs. A3 | -6396 | -35457 to 22665 | No | ns | 0.9695 |
| A2 vs. Controls | -41886 | -70947 to -12825 | Yes | ** | 0.0017 |
| A3 vs. Controls | -35490 | -64551 to -6429 | Yes | ** | 0.0100 |
| | | | | | |
| 50ml | | | | | |
| A0 vs. A1 | -17868 | -46929 to 11193 | No | ns | 0.4127 |
| A0 vs. A2 | -20778 | -49839 to 8283 | No | ns | 0.2653 |
| A0 vs. A3 | -23704 | -52765 to 5357 | No | ns | 0.1568 |
| A0 vs. Controls | -57491 | -86552 to -28429 | Yes | **** | <0.0001 |
| A1 vs. A2 | -2910 | -31971 to 26151 | No | ns | 0.9985 |
| A1 vs. A3 | -5835 | -34896 to 23226 | No | ns | 0.9782 |
| A1 vs. Controls | -39622 | -68683 to -10561 | Yes | ** | 0.0032 |
| A2 vs. A3 | -2926 | -31987 to 26135 | No | ns | 0.9984 |
| A2 vs. Controls | -36713 | -65774 to -7651 | Yes | ** | 0.0072 |
| A3 vs. Controls | -33787 | -62848 to -4726 | Yes | * | 0.0156 |

**Fig. 4.** Tukey's test results for pairwise comparisons of material compositions (24 h)

Similar analyses were done to analyze the data obtained from samples cultured for 48 h in the medium. The results indicated that the material composition had a statistically significant influence on the survival and proliferation of macrophages. Further, the value of p for powder concentration was also less than 0.05, and hence it was concluded that the concentration of the powder also had a statistically significant influence on the macrophages. However, it was seen that the independent variables did not have an interaction effect that was statistically significant. The Tukey's test was performed to further analyze to identify the groups that had a statistical difference between them. Fig. 5 shows the results obtained from the two-way ANOVA test while Fig. 6 and Fig. 7 represent the results obtained from the Tukey's tests.

| | | | | | |
|---|---|---|---|---|---|
| Table Analyzed | CellTiter RAW 48hours | | | | |
| | | | | | |
| Two-way ANOVA | Ordinary | | | | |
| Alpha | 0.05 | | | | |
| | | | | | |
| Source of Variation | % of total variation | P value | P value summary | Significant? | |
| Interaction | 17.77 | 0.1167 | ns | No | |
| POWDER CONCENTRATION | 16.73 | 0.0014 | ** | Yes | |
| MATERIAL COMPOSITION | 29.58 | <0.0001 | **** | Yes | |
| | | | | | |
| ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
| Interaction | 141434023 | 12 | 11786169 | F (12, 40) = 1.649 | P=0.1167 |
| POWDER CONCENTRATION | 133220824 | 3 | 44406941 | F (3, 40) = 6.212 | P=0.0014 |
| MATERIAL COMPOSITION | 235520514 | 4 | 58880128 | F (4, 40) = 8.237 | P<0.0001 |
| Residual | 285926447 | 40 | 7148161 | | |
| | | | | | |
| Number of missing values | 0 | | | | |

**Fig. 5.** Two-way ANOVA results for cytotoxicity test with macrophages (48 h)

| Tukey's multiple comparisons test | Mean Diff. | 95.00% CI of diff. | Significant? | Summary | Adjusted P Value |
|---|---|---|---|---|---|
| 3ml | | | | | |
| A0 vs. A1 | 4919 | -1315 to 11154 | No | ns | 0.1816 |
| A0 vs. A2 | 1422 | -4813 to 7657 | No | ns | 0.9654 |
| A0 vs. A3 | 7574 | 1339 to 13808 | Yes | * | 0.0105 |
| A0 vs. Controls | -426 | -6661 to 5809 | No | ns | 0.9997 |
| A1 vs. A2 | -3497 | -9732 to 2737 | No | ns | 0.5048 |
| A1 vs. A3 | 2654 | -3581 to 8889 | No | ns | 0.7423 |
| A1 vs. Controls | -5345 | -11580 to 889.4 | No | ns | 0.1234 |
| A2 vs. A3 | 6152 | -83.28 to 12386 | No | ns | 0.0547 |
| A2 vs. Controls | -1848 | -8083 to 4387 | No | ns | 0.9143 |
| A3 vs. Controls | -8000 | -14234 to -1765 | Yes | ** | 0.0061 |
| | | | | | |
| 10ml | | | | | |
| A0 vs. A1 | 4551 | -1684 to 10785 | No | ns | 0.2466 |
| A0 vs. A2 | 5808 | -426.3 to 12043 | No | ns | 0.0783 |
| A0 vs. A3 | 3572 | -2663 to 9807 | No | ns | 0.4839 |
| A0 vs. Controls | -1085 | -7320 to 5150 | No | ns | 0.9872 |
| A1 vs. A2 | 1258 | -4977 to 7493 | No | ns | 0.9778 |
| A1 vs. A3 | -978.9 | -7214 to 5256 | No | ns | 0.9913 |
| A1 vs. Controls | -5636 | -11871 to 599 | No | ns | 0.0932 |
| A2 vs. A3 | -2237 | -8472 to 3998 | No | ns | 0.8425 |
| A2 vs. Controls | -6894 | -13128 to -658.9 | Yes | * | 0.0238 |
| A3 vs. Controls | -4657 | -10892 to 1578 | No | ns | 0.2264 |
| | | | | | |
| 30ml | | | | | |
| A0 vs. A1 | 2400 | -3835 to 8635 | No | ns | 0.8058 |
| A0 vs. A2 | 426.1 | -5809 to 6661 | No | ns | 0.9997 |
| A0 vs. A3 | 1633 | -4602 to 7868 | No | ns | 0.9436 |
| A0 vs. Controls | -5634 | -11869 to 600.6 | No | ns | 0.0933 |
| A1 vs. A2 | -1974 | -8208 to 4261 | No | ns | 0.8936 |
| A1 vs. A3 | -766.8 | -7002 to 5468 | No | ns | 0.9966 |
| A1 vs. Controls | -8034 | -14269 to -1799 | Yes | ** | 0.0059 |
| A2 vs. A3 | 1207 | -5028 to 7442 | No | ns | 0.981 |
| A2 vs. Controls | -6060 | -12295 to 174.5 | No | ns | 0.0603 |
| A3 vs. Controls | -7267 | -13502 to -1032 | Yes | * | 0.0153 |
| | | | | | |
| 50ml | | | | | |
| A0 vs. A1 | -192.7 | -6428 to 6042 | No | ns | >0.9999 |
| A0 vs. A2 | -473.1 | -6708 to 5762 | No | ns | 0.9995 |
| A0 vs. A3 | -1288 | -7523 to 4946 | No | ns | 0.9758 |
| A0 vs. Controls | -2013 | -8248 to 4222 | No | ns | 0.8867 |
| A1 vs. A2 | -280.4 | -6515 to 5954 | No | ns | >0.9999 |
| A1 vs. A3 | -1096 | -7331 to 5139 | No | ns | 0.9867 |
| A1 vs. Controls | -1820 | -8055 to 4414 | No | ns | 0.9185 |
| A2 vs. A3 | -815.3 | -7050 to 5419 | No | ns | 0.9957 |
| A2 vs. Controls | -1540 | -7775 to 4695 | No | ns | 0.9541 |
| A3 vs. Controls | -724.6 | -6959 to 5510 | No | ns | 0.9973 |

**Fig. 6.** Tukey's test to identify the statistically differing material compositions (48 h)

The results of the Tukey's test (Fig. 6) revealed that at some concentrations, certain materials differed statistically from the control group. However, there was no difference between the different materials used except in one case (A0 vs. A3 at 3 µl/ml).

| Tukey's multiple comparisons test | Mean Diff. | 95.00% CI of diff. | Significant? | Summary | Adjusted P Value |
|---|---|---|---|---|---|
| **A0** | | | | | |
| 3ml vs. 10ml | 659.2 | -5192 to 6511 | No | ns | 0.9903 |
| 3ml vs. 30ml | 5208 | -643.2 to 11059 | No | ns | 0.0963 |
| 3ml vs. 50ml | 1587 | -4264 to 7438 | No | ns | 0.8857 |
| 10ml vs. 30ml | 4549 | -1302 to 10400 | No | ns | 0.1759 |
| 10ml vs. 50ml | 927.8 | -4923 to 6779 | No | ns | 0.9739 |
| 30ml vs. 50ml | -3621 | -9472 to 2230 | No | ns | 0.3585 |
| **A1** | | | | | |
| 3ml vs. 10ml | 290.4 | -5561 to 6142 | No | ns | 0.9991 |
| 3ml vs. 30ml | 2689 | -3163 to 8540 | No | ns | 0.6108 |
| 3ml vs. 50ml | -3525 | -9376 to 2326 | No | ns | 0.3821 |
| 10ml vs. 30ml | 2398 | -3453 to 8249 | No | ns | 0.6925 |
| 10ml vs. 50ml | -3815 | -9667 to 2036 | No | ns | 0.3132 |
| 30ml vs. 50ml | -6214 | -12065 to -362.3 | Yes | * | 0.0337 |
| **A2** | | | | | |
| 3ml vs. 10ml | 5046 | -805.6 to 10897 | No | ns | 0.1124 |
| 3ml vs. 30ml | 4212 | -1639 to 10064 | No | ns | 0.2322 |
| 3ml vs. 50ml | -308.1 | -6159 to 5543 | No | ns | 0.999 |
| 10ml vs. 30ml | -833.4 | -6685 to 5018 | No | ns | 0.9808 |
| 10ml vs. 50ml | -5354 | -11205 to 497.6 | No | ns | 0.0834 |
| 30ml vs. 50ml | -4520 | -10372 to 1331 | No | ns | 0.1802 |
| **A3** | | | | | |
| 3ml vs. 10ml | -3343 | -9194 to 2509 | No | ns | 0.4289 |
| 3ml vs. 30ml | -732.3 | -6584 to 5119 | No | ns | 0.9868 |
| 3ml vs. 50ml | -7275 | -13126 to -1424 | Yes | ** | 0.0097 |
| 10ml vs. 30ml | 2610 | -3241 to 8462 | No | ns | 0.6331 |
| 10ml vs. 50ml | -3932 | -9784 to 1919 | No | ns | 0.2877 |
| 30ml vs. 50ml | -6543 | -12394 to -691.3 | Yes | * | 0.0232 |
| **Controls** | | | | | |
| 3ml vs. 10ml | 0 | -5851 to 5851 | No | ns | >0.9999 |
| 3ml vs. 30ml | 0 | -5851 to 5851 | No | ns | >0.9999 |
| 3ml vs. 50ml | 0 | -5851 to 5851 | No | ns | >0.9999 |
| 10ml vs. 30ml | 0 | -5851 to 5851 | No | ns | >0.9999 |
| 10ml vs. 50ml | 0 | -5851 to 5851 | No | ns | >0.9999 |
| 30ml vs. 50ml | 0 | -5851 to 5851 | No | ns | >0.9999 |

**Fig. 7.** Tukey's test to identify the statistically differing powder concentrations (48 h)

The results of the second Tukey's test (Fig. 7) revealed that in most cases the use of different powder concentrations did not have a significant impact on the survival of the macrophages except in three cases.

## 2. Analysis for CTB® Cell Viability Assay with MG63 cells

The statistical analysis for the study with MG63 cells was conducted the same way as it was conducted for the cytotoxicity test with macrophages. In this case, the independent variables were material composition and cell culture time. Moreover, it was ensured that the obtained results obeyed the three major assumptions of two-way ANOVA. The results of two-way ANOVA

revealed that the materials did not have a significant difference between them indicating that the materials tested were comparable. However, as expected, there was a significant statistical difference between the fluorescence intensity values obtained from the electrospun membranes seeded for 3 days and the ones seeded for 7 days (Fig. 8). This result was used to conclude that the cells were able to colonize and proliferate with the passage of time.

| | | | | | |
|---|---|---|---|---|---|
| Table Analyzed | CellTiter Fiber Scaffolds Paper March 2017 | | | | |
| | | | | | |
| Two-way ANOVA | Ordinary | | | | |
| Alpha | 0.05 | | | | |
| | | | | | |
| Source of Variation | % of total variation | P value | P value summary | Significant? | |
| Interaction | 14.87 | 0.0036 | ** | Yes | |
| TIME | 71.36 | <0.0001 | **** | Yes | |
| MATERIAL COMPOSITION | 2.101 | 0.4352 | ns | No | |
| | | | | | |
| ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
| Interaction | 1082029222 | 3 | 360676407 | F (3, 16) = 6.801 | P=0.0036 |
| TIME | 5192165512 | 1 | 5192165512 | F (1, 16) = 97.9 | P<0.0001 |
| MATERIAL COMPOSITION | 152842508 | 3 | 50947503 | F (3, 16) = 0.9606 | P=0.4352 |
| Residual | 848553912 | 16 | 53034619 | | |
| | | | | | |
| Number of missing values | 0 | | | | |

**Fig. 8.** Two-way ANOVA results for the electrospun membranes tested with MG63 cells