The Investigation of ZnO/ Poly(vinylidene) Fluoride Nanocomposites for Orthopedic Applications with Improved Mechanical, Piezoelectric, and Antimicrobial Properties

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Abstract

Many studies have shown that piezoelectric materials can be used as bioactively charged surfaces to enhance cell functions and result in tissue regeneration (such as nerve injury repair, bone formation, wound healing, and more). Poly(vinylidene) fluoride (PVDF) and zinc oxide (ZnO) are regarded as potential bone tissue engineering materials because of their many attractive properties including biocompatibility, high piezoelectricity and good mechanical properties. ZnO nanoparticles (NPs) are also well known for their antibacterial properties, in which infection is a growing concern in orthopedics. In this study, PVDF scaffolds doped with crystalline ZnO NPs (termed ZnO/PVDF) were prepared by electrospinning, followed by chemical characterization via Fourier Transform Infrared Microscopy (FTIR). Additionally, their piezoelectric and mechanical properties were also evaluated. In vitro osteoblast (or bone forming cells) assays were performed to determine material cytotoxicity and bone regrowth potential. *Staphylococcus aureus* (SA), *Methicillin-resistant Staphylococcus aureus* (MRSA) and *Escherichia coli* (*E. coli*) bacteria were also seeded and counted to evaluate scaffold antimicrobial properties. Results of this study showed for the first time significantly reduced *E. coli*, *SA* and *MRSA* density on ZnO/PVDF scaffolds when using 2 or 1 mg/ml of ZnO in PVDF composites compared to pure PVDF scaffolds (controls) in 6 hours culture. Compared to controls and non-piezo excited samples, osteoblast density was 30% greater when scaffolds were piezo-excited in 1 and 3 days cell culture. Significantly
decreased bacteria (E. coli, SA and MRSA) density and increased osteoblast density on the piezoelectric stimulated ZnO/PVDF scaffolds demonstrated that these scaffolds have a strong potential for antibacterial orthopedic applications, especially considering that bacteria growth was minimized without using antibiotics and, thus, this approach does not contribute to the growing problem of antibiotic-resistance bacteria troubling medicine today. Moreover, with an increased β phase ratio in PVDF, enhanced mechanical properties also indicated that the ZnO/PVDF scaffolds can be considered as an orthopedic implant material or used for other mechanical and electrical applications with greater efficiency than what may be presently available.

**Key words**: Poly(vinylidene) fluoride, zinc oxide, piezoelectricity, electrospinning, crystal structure, mechanical properties, and orthopedic applications
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1.0 Introduction

1.1. Motivation and background

With an aging world population and an increasing number of sports injuries in many countries, the treatment of orthopedic problems has become a significant challenge in medicine. In the United States, based on data from the National Health Interview Survey, an estimated 52.5 million (22.7 percent) of adults have doctor-diagnosed arthritis, and 22.7 million (9.8 percent) have arthritis and arthritis-attributable activity limitations.\(^1\) In fact, orthopedic problems account for half of all chronic diseases in people over 50 years of age in developed countries, and it is predicted that the percentage of persons over 50 years of age affected by bone diseases will double by 2020\(^2\).

The field of orthopedics encompasses any issue concerning muscles, ligaments or joints, and numerous alternative strategies to accelerate healing and improve function are being actively investigated. Conventionally, surgical implants of various materials including metals, ceramics, and polymers are the main mechanism for treating orthopedic disorders. These materials must satisfy numerous diverse properties, like mechanical properties, such as flexibility, elongation at break, etc., to meet orthopedic requirements of human body tissues \(^3\). Moreover, infection is a crucial issue in orthopedic applications. In the United States, orthopedic implants are associated with an approximate 5% infection rate, representing 100,000 infections per year \(^4\). Thus, in order to help orthopedic patients achieve better recovery and avoid bacterial torment, it is necessary to fabricate new materials that are both biocompatible and antimicrobial.
1.2 Objectives and Scope

The main objective of this study was to develop nano-fiber PVDF scaffolds that exhibit high $\beta$ phase PVDF ratios, enhance osteoblast proliferation via piezoelectric stimulation and resist bacterial growth. Thus, the specific aims of this thesis include:

1. To investigate the morphology of ZnO/PVDF scaffolds with different concentrations of ZnO NPs by using SEM.

2. To enhance the mechanical properties of ZnO/PVDF scaffolds, such as Young’s modulus, maximum load stress and elongation at break, with different concentrations of ZnO NPs.

3. To analyze the possible increased ratio of $\beta$ phase in PVDF by a Fourier Transform Infrared (FT-IR) spectroscopy study.

4. To evaluate the cytocompatibility of ZnO/PVDF scaffolds by human osteoblasts and determine the optimal ZnO concentration for osteoblast proliferation.

5. To examine the osteoblast proliferation rate between ZnO/PVDF scaffolds with and without piezoelectric excitation.

6. To investigate the antibacterial ability of ZnO/PVDF scaffolds to E. coli, SA and MRSA, and analyze the antimicrobial function of the piezoelectric effect.
2.0 Critical Literature Review

2.1 Challenges of Orthopedic Implants

2.1.1 Mechanical Properties

To date, orthopedic implants have been made of various materials; however, no single type of material possesses all of the necessary mechanical properties required for optimal orthopedic therapies \[^5\]. Siegler et al. \[^6\] determined the mechanical properties of a collateral human ankle joint ligament, finding an elastic modulus ranging from 90 to 500 MPa and an elongation at break ranging from 12\% to 25\%. This result showed that the ankle ligament was rigid but flexible, able to tolerate a high amount of pressure with a considerable strain ratio in the human body; matching such properties in metallic and ceramic material is difficult, if not impossible. Even if these materials possess a high modulus, a low elongation ratio still restricts their application as a ligament replacement. Similarly, most polymers face a severe modulus and stress mismatch to natural ligament tissues.

2.1.2 Infection Resistances

Infection is another crucial issue in orthopedic applications \[^7,8\]. Millions of orthopedic implants are used each year, and a significant proportion of each type of implant is colonized by bacteria and causes an implant-related infection \[^4\]. In many orthopedic infection cases, \textit{Staphylococcus aureus (SA)} is a major factor, as shown in Figure 1. For example, \textit{SA} has been the dominant pathogen for all classes of osteomyelitis, accounting for 45\% of orthopedic infections \[^9\].
Figure 1. Prevalence of the five most frequent pathogens as a function of the origin of the orthopedic infection in a collection of 272 clinical isolates obtained from 242 patients in the period between 2007 and 2011. (K: Knee; H: Hip; IF: Internal fixation; EF: External fixation; No MD: No Medical device) (adopted from [4])

2.2 Biomaterial Factors Affect Bone Regeneration

Factors that influence bone reconstruction are varied. From previous studies, many properties (such as biocompatibility (especially inflammatory), mechanical properties, surface topography, energy, antimicrobial abilities, etc.) that may affect bone cell function and stimulate bone construction have been investigated [10-16].

2.2.1 Biocompatibility Effect

Biocompatibility is the main concern for orthopedic biomaterial selection. Materials with low biocompatibility cause diverse issues that inhibit tissue regeneration and even kill the tissue, such as inflammation [10]. Inflammation causes mass fibroblast generation and functions, isolating the target tissue and biomaterials and even leads to corrosions of implants, as seen in Figure 2 [11].
2.2.2 Mechanical Properties Effect

As mentioned in 2.1.1, orthopedic implants have to meet high mechanical requirements. Materials with lower mechanical properties are not suitable for bone replacement, as these materials cannot support the body as effectively and can be easily deformed or degraded \[^{12}\].

2.2.3 Surface Energy Effect

Surface energy is another factor that may influence bone cell behavior. In a previous study, different surface energy can make cell adhesion different \[^{13}\]. In addition, there are many bio-electric material that can help cellular functions \[^{14}\]. Therefore, surface energy may also affect cell functions by acting with such reactions. Moreover, in the previous study, small currents were even used to stimulate bone cell growth \[^{15}\]. Thus,
it is highly recommended to select a material that can be surface energy modified or can generate energy itself for bone tissue formation.

2.2.4 Surface Topography Effect

Different kinds of material surface topographies have been used to stimulate bone cell functions because different topographies suggest different micro-environments. Lyndon et al [16] reviewed the role of surface topography in creating and maintaining bone at a titanium endosseous implant. They concluded that changed titanium implant surface topography improved bone-to-implant contact and the mechanical properties of the enhanced interface, and changed bone cell behavior as a result, as shown in Table 1.

Table 1. Different surface topographies and their effects on bone-to-implant contact at titanium endosseous implants (adopted from [16])

<table>
<thead>
<tr>
<th>Surface modifications</th>
<th>Model</th>
<th>Bone-to-implant contact</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPS vs HA</td>
<td>Canine</td>
<td>HA&gt;TPS</td>
</tr>
<tr>
<td>TPS vs SLA</td>
<td>Canine</td>
<td>SLA&gt;TPS</td>
</tr>
<tr>
<td>Mesh vs etch vs GB</td>
<td>Rabbit</td>
<td>Etch&gt;GB&gt;mesh</td>
</tr>
<tr>
<td>TPS vs SLA vs EP</td>
<td>Minipig</td>
<td>SLA&gt;TPS&gt;EP</td>
</tr>
<tr>
<td>GB vs etch vs HA</td>
<td>Minipig</td>
<td>HA&gt;TPS&gt;etch</td>
</tr>
<tr>
<td>GB vs Ti₆Al₄V vs GB CoCr</td>
<td>Rabbit</td>
<td>Ti₆Al₄V &gt;CoCr</td>
</tr>
<tr>
<td>GB vs etch vs machined Ti</td>
<td>Rabbit</td>
<td>No difference</td>
</tr>
<tr>
<td>GB Ti₆Al₄V vs EP Ti₆Al₄V</td>
<td>Rabbit</td>
<td>GB Ti₆Al₄V = EP Ti₆Al₄V</td>
</tr>
<tr>
<td>75 µm GB vs machined</td>
<td>Rabbit</td>
<td>75 µm GB = machined</td>
</tr>
<tr>
<td>25 µm GB vs 75 µm GB vs machined</td>
<td>Rabbit</td>
<td>75 µm GB &gt; 25 µm GB &gt; vs machined at 1 y</td>
</tr>
<tr>
<td>25 µm GB vs 75 µm GB</td>
<td>Rabbit</td>
<td>75 µm GB &gt; 25 µm GB at 12 wk</td>
</tr>
<tr>
<td>Etched vs machined</td>
<td>Sheep</td>
<td>No difference</td>
</tr>
<tr>
<td>25 µm GB vs 25 µm TiO₂</td>
<td>Rabbit</td>
<td>25 µm GB = 25 µm TiO₂</td>
</tr>
<tr>
<td>25 µm GB vs 250 µm GB</td>
<td>Rabbit</td>
<td>25 µm GB &gt; 250 µm GB</td>
</tr>
<tr>
<td>25 µm TiO₂ vs 75 µm GB vs machined</td>
<td>Rabbit</td>
<td>TiO₂ &gt; machined</td>
</tr>
<tr>
<td>TiO₂ vs machined</td>
<td>Rabbit</td>
<td>TiO₂ vs machined</td>
</tr>
<tr>
<td>TiO₂ vs machined</td>
<td>Canine</td>
<td>TiO₂ vs machined</td>
</tr>
<tr>
<td>Etch vs GB vs machined</td>
<td>Rat</td>
<td>Etch = GB = machined</td>
</tr>
</tbody>
</table>

2.2.5 Antimicrobial Effect

Antibacterial ability is an essential function that orthopedic materials should possess to protect bone growth. As mentioned in section 1.2, infection is a severe issue in orthopedic surgeries; once infected, the bone tissue will be killed by bacteria causing significant discomfort to the patient. Conventional antimicrobial mechanisms generally fall within one of four ways\textsuperscript{[17]}: inhibition of cell wall synthesis, inhibition of protein synthesis, alteration of cell membrane and inhibition of nucleic acid synthesis. Antibiotics like penicillin, cephalosin function in this way. Also, many nanoparticles can inhibit bacteria growth or kill bacteria because they can form a positive charge in water. Since almost all bacteria have negative charges on the bacteria surface, cationic NPs can combine with a bacteria surface and inhibit bacteria by interrupting bacterial activities. Moreover, there is another novel method that can be used for antibacterial applications. Antibacterial surfaces (or topographies) are capable of repelling bacterial cells if created in the right way, preventing their attachment or even killing cells that do come into contact with the surface physically or chemically\textsuperscript{[18]}.

2.3 3D Scaffolds in Tissue Engineering

It is clear that nearly all of tissues in the human body are three dimensional, such as bone, vessels and muscles. Unlike 2D biomaterials, which can only affect the first few cell layers in proximity to the implant, 3D scaffold topographies offer a significant advantage because of their increased influence on multiple cell layers\textsuperscript{[19]}. Therefore, over decades, tissue engineering applications commonly encompass the use of 3D biomaterials (especially scaffolds) to provide a desired micro-environment, and interact with cells to regenerate damaged tissues. Moreover, different materials were
used to fabricate 3D scaffolds that serve to imitate the actual in vivo micro-environment where cells interact and behave according to the mechanical cues obtained from the surrounding 3D environment\textsuperscript{[20]}.

Scaffolds can be categorized by criteria such as the fabricating materials, their morphologies, their applications and others. Regarding the morphological criterion, scaffolds can be considered as two main types: porous scaffolds and fibrous scaffolds.

Porous scaffolds are scaffolds that have high porosity, like Figure 3 \textsuperscript{[21]} shows. According to Karageorgiou et al \textsuperscript{[22]}, porosity (the number of pores) and pore size, both at the macro- and microscopic level, are important morphological properties for bone regeneration. Fibrous scaffolds are scaffolds formed by fibers. A small piece of a fibrous scaffold contains millions of fibers, which range in size (or diameter) from the micro- to nanometer level. From research performed by Badami et al \textsuperscript{[23]}, the surface topography introduced by the PLLA electrospun fibers (as seen in Figure 4) affects cell morphology and cell proliferation because of the network structure of the scaffolds.

![Figure 3. Morphology of a HA porous scaffold (adopted from \textsuperscript{[21]})](image)
2.4 Piezoelectricity and Orthopedics

2.4.1 Piezoelectric Materials and Its Effects on Cell Functions

Piezoelectricity is the phenomenon that occurs when solid materials generate electrical charge in response to deformation \cite{24,25}. Recently, the use of biocompatible piezoelectric materials (such as ZnO, barium titanate (BaTiO$_3$) and poly(vinylidene) fluoride (PVDF)) to enhance cell functions has become a popular field of study. Bone is a piezoelectric material that exhibits electricity \cite{26-29}, which can stimulate various biochemical reactions, and energy conversion can be achieved in the process during these biochemical reactions \cite{14}. Electricity in biosystems (bio-electricity) also promotes growth factor activity and the formation of an extracellular matrix (ECM), which can induce bone reconstruction \cite{30}.

To investigate piezoelectricity as a type of bio-electricity, research has been done to investigate the effect of piezoelectric materials on tissues. A previous study reported that bone-like crystals formed on BaTiO$_3$ in vitro can lead to calcium phosphate deposits after being polarized \cite{31}. Neuron cell adhesion and differentiation can also be induced on piezoelectric fiber scaffolds \cite{32}. In addition, Guo et al. showed stimulated fibroblast functions on polyurethane (PU)/PVDF scaffolds, suggesting that both the migration and proliferation rate of fibroblasts can
be induced on PU/PVDF piezoelectric scaffolds \[^{33}\]. Lastly, Bhang et al. used ZnO nanorod-based piezoelectric dermal patches to stimulate wound healing \[^{34}\]. These previous studies were the main motivation for this project in investigating the orthopedic applications of ZnO/PVDF scaffolds.

Moreover, as mentioned in section 2.2.5, almost all bacteria have negative charges on their membranes, and these negative charges can be attracted by a cationic surface. Hence, the cationic surface formed by piezoelectric material should be able to attract bacteria, and then even kill bacteria by special topographies of a material surface or doped nanoparticle. These hypotheses were the motivation in investigating piezoelectric materials for antimicrobial applications here.

### 2.4.2 Piezoelectric Properties of PVDF

Poly(vinylidene) fluoride (PVDF) is a commonly used piezoelectric semi-crystalline polymer. The piezoelectric properties of PVDF were first discovered by Kawai et al. \[^{35}\] in 1969. PVDF has at least four polymorphs: α, β, γ and δ \[^{36, 37}\]. Among them, the α phase is the most common crystallization phase; however, the β structure is the most desired structure in this research because it exhibits a strong piezoelectric property. Therefore, in order to obtain an excellent piezoelectric material, it is necessary to fabricate PVDF materials with a high percentage of β phase.

The β phase PVDF can be formed by conventional methods, like mechanical stretching and poling the α phase PVDF \[^{38}\]. Because the α phase PVDF crystalline regions will align such that all dipole elements cancel each other, and no electric field will be formed. When poling or stretching the α phase PVDF properly, fluorine will be arranged on one side and hydrogen will be arranged on the other side, thus forming
the β phase of PVDF. This structure causes a dipole moment formed in a stacked direction inside the PVDF β phase crystalline regions [39], as Figure 5 shows.

When force is applied to this multi-layer polymer chain region, as shown in Figure 6, the local dipole distributions change and an electric field is induced in the stack. The induced electric field accumulates charges at two sides of the film, thereby demonstrating piezoelectricity.

![Figure 5. Chemical structure of the α phase and β phase PVDF: (a) α phase PVDF structure and (b) β phase PVDF structure (adopted from [39])](image)

![Figure 6. Schematic illustration of the β phase crystalline region (adopted from [39])](image)

### 2.5 High Ratio β Phase PVDF Fiber Preparation: Electrospinning

Electrospinning is a fiber production method that uses an electrical field and force to draw charged threads of polymer solutions or melts, stretching polymers into fibers. It is a promising method used to construct fused-fiber biomaterial scaffolds for tissue
Moreover, for PVDF, it is difficult to get a 3D structure of the β phase PVDF \cite{30} using conventional methods (like stretching or poling). Previous studies have shown that electrospinning can be used to obtain PVDF with a β phase and 3D structure \cite{36, 37, 42}. Thus, electrospinning can be considered as a potential method to obtain both a 3D structure and β phase PVDF. The schematic structure of electrospinning is shown in Figure 7 \cite{43}.

![Figure 7. Schematic Structure of Electrospinning (adopted from \cite{43})](image)

### 2.5.1 Concentration Requirement of Electrospun Solution

Conventionally, electrospinning requires the proper concentration of a polymer electrospun solution to ensure fiber quality because a low concentration electrospun solution will cause bead structure formation and reduce chemical and physical properties of the fibers \cite{44, 45, 46}. As shown in Figure 8, with a polystyrene (PS) concentration increase, the bead density decreased. In this situation, for PVDF fibers, the scaffolds require a really high concentration (always over 20\%), when using PVDF
MW~275000, needle 23G) to reduce bead formation. However, this significantly raises the cost of production.

Figure 8. Optical microscope images (5× enlargement) of PS fibers electrospun from THF as a function of PS concentration: (a) 18 wt %; (b) 20 wt %; (c) 25 wt %; (d) 28 wt %; (e) 30 wt %; and (f) 35 wt %. (Adopt from [45])

2.5.2 Charge Density Effect

Charge density is a measure of electrical charge per unit volume of space, in one, two or three dimensions. In the electrospinning process, the charge density carried by the moving jet (Coulomb/liter), correlates with the formation of beaded fibers. From previous studies, the increased charge density of the polymer solution caused a greater
repulsion and a greater bending instability during electrospinning, and therefore stretched the fibers to thinner fibers with a smaller diameter and eliminated the total number of beads \cite{47,48}, just like Figure 9 showed.

![Figure 9](image)

Figure 9. Variation of beaded fibers as net charge density changed due to the addition of NaCl. Charge density: (a) 1.23 C/l, (b) 1.77 C/l, (c) 3.03 C/l, (d) 6.57 C/l, (e) 8.67 C/l, and (f) 28.8 C/l (The electrical field was 0.7 kV/cm. Weight fraction of PEO was 3.0%. The length of the horizontal edge of each of the images was 20 microns). (adopted from [47])
2.6 Advantaged of ZnO additives

2.6.1 Electrospun Fiber Quality Improvement

In order to solve the bead problem, ZnO NPs were added into the electrospun solution because they can enhance the conductivity of the electrospun solution, consequently enhancing charge density during electrospinning. The increased charge density of the polymer solution then produces thinner fibers, eliminating the total number of beads, as mentioned in Section 2.4.2.

2.6.2 Antibacterial Abilities Improvement

Infection is crucial issue in orthopedic applications \[49,50\]. Thus, even though a 3D and high $\beta$ phase fraction of PVDF showed good cytocompatibility with osteoblasts in vitro \[36,51\], poor mechanical properties, lack of antibacterial properties, and a high production cost still limit its applications in orthopedic applications. Earlier studies have shown that ZnO NPs can resist several kinds of bacteria, including \textit{SA}, \textit{MRSA}, and \textit{E. coli} both in vitro and vivo \[52-57\]. For this reason, the antibacterial properties of ZnO/PVDF scaffolds were also investigated here. On this basis, ZnO NPs were assumed to be good additives to improve not only the piezoelectric property of PVDF but also the antibacterial properties of PVDF fibers.

2.6.3 Mechanical Properties Enhancement

In recent years, nanocomposites have attracted a great deal of focus due to their ability to improve the mechanical ability of polymers \[58,59\]. In a previous study, Li et al. \[60\] used ZnO NPs to enhance the mechanical properties of a polyurethane material. Research results (as shown in Figure 1.) showed that with the addition of ZnO NPs (from 0 to 2 wt%), the Young’s modulus and tensile strength of the PU composite
films increased from 517 to 710 MPa and enhanced from 8.6 to 17.8 MPa, respectively. Thus, ZnO NPs are considered as a good additive candidate to improve the mechanical properties of scaffolds in addition to improve piezoelectric and antimicrobial properties, as discussed in section 2.5.2.

Figure. 10. Tensile stress–strain curves of PU films with different ZnO content (wt%). (adopted from [60])

2.7 Summary

Orthopedic problems and infections followed by orthopedic surgeries has become a large problem for the 21st century \cite{1, 2}. Therefore, the development of an orthopedic implant with enhanced mechanical, piezoelectricity, biocompatibility and antibacterial properties is necessary to help patients reduce orthopedic pain. Owing to the advantages of construction and special electrical properties, 3D piezoelectric scaffolds have become a favored type of material for bio-applications, because piezoelectricity can increase biological electricity to stimulate cell functions. PVDF should become a highly recommended piezoelectric biomaterial for its good piezoelectric properties.
Moreover, a good way to fabricate 3D PVDF scaffolds is through electrospinning. Because of the limitations of electrospinning and unclear antibacterial properties of pure PVDF, ZnO is considered to be a good additive in PVDF scaffolds. ZnO can impart antimicrobial properties, mechanical improvement and conductivity enhancement. Therefore, an electrospun ZnO/PVDF scaffold was fabricated in this study and characterized via mechanical tests and other techniques. In vitro osteoblast assays were also performed to determine material cytotoxicity and initial bone reconstruction potential. In addition, to evaluate the antimicrobial properties of the scaffold, *Staphylococcus aureus* (SA), *Methicillin-resistant Staphylococcus aureus* (MRSA) and *Escherichia coli* (*E. coli*) were separately seeded and counted.
3.0 Material and Methods

3.1 Sample Preparation

PVDF pellets (~27000 MW, 677450-5G, Sigma-Aldrich) were dissolved in acetone/dimethylformamide (DMF) (v/v=3/2) at a concentration of 18% (w/v, g/ml). The solution was stirred at 55 °C for 12 hours. ZnO nanoparticles (<50 nm Sigma-Aldrich) were mixed with the solution at a concentration of 0.5 mg/ml, 1 mg/ml or 2 mg/ml. After mixing the solution, it was sonicated for 1 hour. The ZnO/PVDF solution was then transferred into a 20(24) ml polypropylene syringe. The syringe was connected to a metering pump (New Era, NE-300) and a 22G needle. The metering pump was used to maintain a constant flow rate at 2 ml/hr. A positive high voltage power supply (PowerBright, VC-2000V) at 15 KV was applied between the solution and the aluminum foil collecting plate in an electrospinning machine (Inoveso, Doublespinner).

3.2 Characterization

3.2.1 Transmission Electron Microscopy (TEM)

Transmission electron microscopy (TEM, JEOL JEM 1010) was used to measure the morphology of the ZnO NPs. The NPs were dissolved in DI water at a concentration of 50 µg/ml. The ZnO NPs solution was dropped onto a copper grid (Electron Microscopy Science, CF300-Cu) then the grid was placed in ambient air until the liquid (DI water) evaporated completely. After the liquid evaporated, the grid was transferred into the TEM microscope and scanning parameters were set at a 80 kV high voltage and 50000x magnification.
3.2.2 Dynamic Light Scattering (DLS)

To measure the dimensions of the ZnO NPs, commercial ZnO NPs were purchased as described above and dispersed in the DI water at a concentration of 200 µg/ml. Before the DLS measurements, the ZnO solution was ultra-sonicated for 30 min. The sonicated ZnO NP solution was added into 4.5 ml disposable UV-grade methacrylate cuvettes (Sigma-Aldrich, Z188018) and characterized by DLS using the same scanning parameters as those of pure water.

3.2.3 Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM, Hitachi S4800, Japan) was used to investigate scaffold morphology. Scaffolds were coated with 5 nm of platinum and viewed using an accelerating voltage of 3 kV, current 10 µA and a working distance of ca. 8 mm. Image J (National Institutes of Health, MD, USA) software was used to calculate the average fiber diameter of the scaffolds. Energy-dispersive X-ray spectroscopy (EDS) was also used for chemical verification of the ZnO/PVDF scaffolds.

3.2.4 Fourier Transform Infrared (FTIR) Microscopy

FTIR microscopy (Perkin Elmer Spectrometer) was performed on the electrospun scaffolds for pure PVDF and PVDF at different ZnO NP concentrations. The samples were scanned a total of 30 times for all samples from 400 to 1500 cm\(^{-1}\). Several equations were used to calculate the relative fraction of β phase present in each sample \(^{[36, 61-64]}\) (n = 3 per group). Assuming these absorption bands follow the Beer–Lambert law with absorption coefficients of \(K_α = 6.1 \times 10^4\) and \(K_β = 7.7 \times 10^4\) cm\(^2\) mol\(^{-1}\), and using characteristic absorption bands of the α and β phases at 531 and 840
cm$^{-1}$, respectively, the fraction of the $\beta$ phase can be calculated by the following equation:

$$A = 2 - \log_{10}(\%T) \quad (1)$$

$$F(\beta) = \frac{X_\beta}{X_\beta + X_\alpha} = \frac{A_\beta}{1.26A_\alpha + A_\beta} \quad (2)$$

where $\%T$ and $A$ are the transmittance and absorbance of FTIR; $A_\alpha$ and $A_\beta$ correspond to absorption bands at 531 and 840 cm$^{-1}$; $X_\alpha$ and $X_\beta$ are the crystalline mass fractions of the $\alpha$ and $\beta$ phases, respectively.

### 3.3 Mechanical Properties Tests

The mechanical properties of the prepared scaffolds were measured using the universal testing machine Expert 5000 (Admet Inc. MA. Norwood USA). The force transducer was made using the MTEST Quattro (Admet Inc. MA. Norwood USA.). The crosshead speed was 5 g/sec during the tests. The scaffolds were cut into 10 mm x 30 mm rectangular shape and the average thickness of the scaffolds was 50±3 µm. Different parameters including the modulus of elasticity (kPa), elongation at break (%) and maximum load (N) were determined.

### 3.4 Contact Angle Analysis

Water contact angles were determined using a drop shape analysis system (SEO Phoenix300, Korea). The contact angle from 1 mL sessile droplets of double distilled water (5 s after being placed on the surface) was measured for all samples at room temperature. At least three measurements were carried out for every sample.
3.5 Piezoelectricity Detection

In order to prove that the ZnO/PVDF is piezoelectric, a simple cell (battery) was built to detect the piezoelectricity that was generated by the ZnO/PVDF scaffold during loading. For this, two pieces of the 1 cm x 2.5 cm aluminum foil (Fisherbrand 01-213-101) were used as the electrodes. The ZnO/PVDF scaffolds were cut into 1 cm x 2 cm and adhered to the aluminum foil with carbon conductive tape (ELECTRON MICROSCOPY SCIENCES, 76762-01). Each aluminum electrode had a 0.5 cm uncovered place and were linked with a digital multi-meter (Crenova MS8233D). The cell was bent with force, and piezoelectricity was measured by a linked electrical meter.

3.6 In Vitro Cell Studies

3.6.1 Cell Culture

Human osteoblasts (Lonza CC-2509) were seeded and cultured at 37°C in 5%/95% CO₂/air in a mixed medium consisting of Osteoblast Basal Medium (Promocell GmbH C27015) supplemented with 10% Osteoblast Supplement Mix (Promocell GmbH C39615), and 1% Penicillin-Streptomycin (Sigma-Aldrich, P4333-100ML).

3.6.2 Cell Proliferation Tests without Piezoelectric Treatment

The ZnO/PVDF scaffolds were cut into 1 cm² squares, adhered by SEM carbon tape and attached onto glass coverslips (Corning BioCoat 12 mm). The samples were then sterilized in UV light for 60 min and later rinsed with Dulbecco’s Phosphate Buffered Saline (PBS) (Sigma Aldrich, D537) for 10 minutes. Prior to cell seeding, scaffolds were transferred to a non-adherent, 24-well polypropylene tissue culture plate (Corning REF 353047). Human osteoblasts were seeded at 15,000 cells/cm² onto 1
cm² samples and cultured in 24 well tissue culture plates (Corning 353047) for 1 to 3 days. A cell proliferation assay kit (Colorimetric) (MTS) (Promega G3581) was used to assess cell growth and the assay solution was added into the wells at a ratio of 1:5 (V_{MTS}/V_{Medium}) (volume) for 4 hours with the medium removed to determine the optical density (OD) at a 490 nm wavelength. A standard curve was created to correlate OD to cell number.

### 3.6.3 Cell Proliferation Test with Piezoelectric Treatment

The scaffolds were cut into 0.49 cm² squares and attached by a silicone tissue adhesive onto 24-well silicone well plates (WPI, CS-MCFX-24). The samples and silicone well plate were then sterilized in UV light for 60 min and later rinsed with PBS for 10 minutes. Prior to cell seeding, the well plates with scaffolds attached were transferred into a mechanical stretching machine (MSM) (WPI, CS-MECHANO-FX) for piezoelectric stimulation. The mechanical stretching stimulation was set at a 1 Hz frequency and 10 mm strain to avoid the vibration of well plate and reach a high ratio of deformation. Human osteoblasts were then seeded on these piezoelectric excited scaffolds (adhered on the silicone well plate) at 10,000 cells/cm². The machine was moved into a standard cell culture incubator for 1 to 3 days. MTS assays were added into the cell seeded well at a ratio of 1:5 (MTS/Medium) for 4 h and the medium was removed to test the OD at a 490 nm wavelength. A standard curve was created to correlate ABS to cell number.

### 3.6.4 Antibacterial Tests without Piezoelectric Treatment

ZnO/PVDF scaffolds (fabricated with different ZnO concentrations) were cut into 1 cm² square and adhered on 12 mm diameter glass coverslips. The samples were then
sterilized in UV light for 60 min and later rinsed with Dulbecco’s Phosphate Buffered Saline (PBS) (Sigma Aldrich, D537) for 10 minutes. *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*SA*) and *Methicillin-resistant Staphylococcus aureus* (*MRSA*) were then seeded on 1 cm$^2$ scaffolds in a 24 Non-Tissue Culture Well plate (Corning, REF 351143) at 1 x 10$^6$ cells/ml and cultured for 24 h. The scaffolds were taken out, put into a 3 ml PBS solution and ultra-sonicated for 5 min. The sonicated solutions were diluted 1000 times, and the diluted solutions were dropped on an agar plate 3 times separately (each time 10 µL) to form colony groups. The seeded agar plates were cultured in the incubator for 12 h and taken out for colony counting (n=3).

### 3.6.5 Antibacterial Tests with Piezoelectric Treatment

ZnO/PVDF scaffolds (fabricated with different ZnO concentrations) were cut into 0.49 cm$^2$ squares and adhered on the bottom of 24 silicone well plates (WPI, CS-MCFX-24) by 5 µL of a tissue adhesive (Urocare, Uro-Bond III 5000 015). After adherent, the scaffolds with silicone well plates were sterilized in UV light for 60 min and later rinsed with Dulbecco’s Phosphate Buffered Saline (PBS) (Sigma Aldrich, D537) for 10 minutes. *E. coli*, *SA* and *MRSA* at a 1 x 10$^6$ cells/ml concentration were then seeded onto 1 cm$^2$ scaffolds in the silicone well plate and were cultured for 6 h at 37 °C in 5%/95% CO$_2$/air in 30 g/L Tryptic Soy Broth (TSB) medium. Prior to cell seeding, the silicone well plates, with scaffolds attached, were transferred into the MSM for piezoelectric stimulation. The mechanical stretching stimulation was set at a 1 Hz frequency and 10 mm strain to avoid well plate shaking and to reach a high deformation ratio. As a comparison, the same bacteria were seeded in the silicone well plate and cultured under the same environment (without dynamic stretching). After 6 h of culture, the scaffolds and silicone bottom were cut out, put into a 3 ml
PBS solution and ultra-sonicated for 5 min. 10 µl of the sonicated solutions were dropped on an agar plate 3 times for standard bacteria colony counting assays.

3.7 Statistical Analysis

Window Microsoft Excel 2016 was used for statistical analysis of all the quantitative data. Results are expressed as mean ± standard deviation (S.D.). The statistical analysis parameters were set at tail 1 and pair 1 to calculate a significant difference.
4.0 Results

4.1 TEM and DLS Characterization

The TEM picture (Figure 11) showed that most of the ZnO NPs had an average size of 35-50 nm. The ZnO NPs aggregated easily and formed large clusters. Figure 12 shows the diameter distribution of ZnO NPs as measured by DLS. The diameter of the ZnO NPs were about 40-60 nm (with an average diameter of 42 nm) in Figure 11, while a few ZnO NPs agglomerated and formed large clusters (diameter > 200 nm), which matched the TEM results and the ZnO NP datasheet provided by the commercial supplier.

![Figure 11. TEM image of the ZnO NPs](image)
4.2 SEM Characterization

SEM images of the ZnO/PVDF scaffolds are shown in Figure 13 when fabricated at ZnO NP concentrations from 0 to 2 mg/ml (based on the 18%wt PVDF electrospun solution). The fiber size distribution for each scaffold was determined by analyzing 50 fibers from various SEM images of the scaffolds at different ZnO concentrations. The calculation showed that the average diameter decreased from 360 nm to 240 nm, when adding ZnO NPs into a PVDF solution from 0 to 2 mg/ml. The effect of ZnO NPs on the formation of beads was also investigated. The bead number for each scaffold was determined from at least 10 images each with an area of 40,000 µm$^2$. Results in Table 2 showed that by adding ZnO NPs into an electrospun solution from 0 to 1 and 2 mg/ml, the bead density on the scaffold reduced significantly, from 1761 to 432 and 32 beads/mm$^2$, respectively.

<table>
<thead>
<tr>
<th>ZnO concentration (mg/ml)</th>
<th>0</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bead Density (Beads/mm$^2$)</td>
<td>1762 ± 217</td>
<td>432 ± 73</td>
<td>32 ± 13</td>
</tr>
</tbody>
</table>
Figure 13. SEM images of ZnO/PVDF scaffolds fabricated from the 18% PVDF electrospun solution of: (a) 0 mg/ml ZnO NPs, (b) 1 mg/ml ZnO NPs and (c) 2 mg/ml ZnO NPs.
Figure 14 shows an SEM image of the PVDF scaffold with 1mg/ml of ZnO NPs. The images indicated that the ZnO NPs agglomerated together and anchored on the surface of the fibers. EDS (Fig. 15) was obtained from the SE-SEM images (Fig. 14). The tested area showed a sharp oxygen peak at $K_{\alpha} = 0.525$ keV and a Zn peak at $L_{\alpha} = 1.01$ keV, $K_{\alpha} = 8.63$ keV. Figure 15 confirms that the portions anchored on the fiber surface were agglomerated ZnO NPs.

![SEM image of ZnO NPs anchored on the PVDF fibers](image)

**Figure 14. SEM image for ZnO NPs anchored on the PVDF fibers**

![SEM-EDS analysis of the ZnO/PVDF fibers](image)

**Figure 15. SEM-EDS analysis of the ZnO/PVDF fibers.**
4.3 FTIR Analysis

The FT-IR spectra recorded in the spectral range of 500–1500 cm\(^{-1}\) for pure PVDF scaffolds as well as PVDF scaffolds at ZnO NP concentrations of 0.5 mg/ml, 1 mg/ml and 2 mg/ml (based on the electrospun solution) are shown in Figure 16. The FTIR spectrum of the pure PVDF scaffolds exhibited \(\alpha\) phase peaks at 531, 614, 763, 796, 870 and 970 cm\(^{-1}\) and \(\beta\) phase peaks at 510, 840 and 1278 cm\(^{-1}\) (Figure 16). ZnO/PVDF scaffold-electrospun fibers, fabricated at 15 kV, also showed characteristics of an \(\alpha\) phase with peaks at 531, 614, 763, 796, 870 and 970 cm\(^{-1}\) and a \(\beta\) phase with peaks at 510, 840 and 1278 cm\(^{-1}\), exhibiting no differences from the control. However, when comparing the \(\alpha\) and \(\beta\) peaks intensities of pure PVDF scaffolds and the ZnO/PVDF nanocomposites, PVDF scaffolds with ZnO NP additives were observed at higher peak intensities than pure PVDF scaffolds. In addition, when the transmittance spectrum was converted to the absorbance spectrum by equation (1), the relative \(\beta\) fraction of PVDF can be estimated by equation (2). From Figure 17, the calculated results showed that the addition of ZnO NPs helped change the crystal structure of the PVDF scaffolds. The PVDF \(\beta\) phase ratio was also enhanced, with an increasing ZnO NP concentration.

Figure 16. FTIR transmittance spectra for ZnO/PVDF scaffolds
Figure 17. PVDF β phase fraction at different ZnO NPs concentrations. Values are mean ± STDEV; N=3.

4.4 Mechanical Analysis

The tensile stress–strain curves for ZnO/PVDF scaffolds are shown in Fig. 18 with the calculated mechanical parameters listed in Table 3. It can be seen that the Young’s modulus, maximum load and elongation to failure increased first with an increase in ZnO NP concentration. However, when the ZnO concentration increased up to 2 mg/ml, the Young’s modulus, maximum load and elongation to failure decreased as a consequence of ZnO NP agglomeration. The optimal ZnO NP concentration for orthopedic applications in terms of Young’s modulus (109.2 ± 7.9 MPa), maximum load (4.8 ± 0.4 kN) and elongation to failure (140.16 ± 4.11%) was achieved at 1 mg/ml.
Figure 18. (a) Stress-strain curve for the PVDF scaffolds at different ZnO NP concentrations. (b) Zoom in Stress-strain curve for the PVDF scaffolds at different ZnO NP concentrations in 1% strain.
Table 3. Mechanical properties of PVDF scaffolds with different concentration of ZnO NPs

<table>
<thead>
<tr>
<th>ZnO NPs Concentration (mg/ml)</th>
<th>Modulus of Elasticity (MPa)</th>
<th>Elongation at Break (%)</th>
<th>Maximum Load (kN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>19.9 ± 2.7</td>
<td>89.76 ± 3.53</td>
<td>1.13 ± 0.12</td>
</tr>
<tr>
<td>0.5</td>
<td>73.7 ± 7.4</td>
<td>129.92 ± 4.47</td>
<td>4.29 ± 0.34</td>
</tr>
<tr>
<td>1</td>
<td>109.2 ± 7.9</td>
<td>140.16 ± 4.11</td>
<td>4.80 ± 0.41</td>
</tr>
<tr>
<td>2</td>
<td>75.8 ± 8.3</td>
<td>100.19 ± 3.16</td>
<td>3.92 ± 0.37</td>
</tr>
</tbody>
</table>

4.5 Contact Angle Analysis

The result of contact angles of ZnO/PVDF scaffolds are shown in Figure 19. The contact angle of 0 mg/ml, 0.5 mg/ml, 1 mg/ml, 2 mg/ml ZnO in PVDF scaffolds were 126.3°, 125.6°, 126.6°, 126.2° respectively, which were not statistically different.
Figure 19. DI water droplet on the ZnO/PVDF scaffolds: (a) 0 mg/ml ZnO, (b) 0.5 mg/ml ZnO, (c) 1 g/ml ZnO and (d) 2 mg/ml ZnO.

4.6 Piezoelectricity Detection

The piezoelectric responses of ZnO/PVDF scaffolds were tested, estimated and listed in Table 4. ZnO/PVDF scaffolds at all ZnO concentrations (ranging from 0 to 2 mg/ml) exhibited piezoelectricity.

Table 4. Piezoelectric Response of ZnO/PVDF Scaffolds with Force Stimulation

<table>
<thead>
<tr>
<th>ZnO concentration (mg/ml)</th>
<th>0</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Piezoelectric Response (mV)</td>
<td>42.2 ± 7.3</td>
<td>46.7 ± 7.1</td>
<td>51 ± 10.2</td>
<td>51.3 ± 9.3</td>
</tr>
</tbody>
</table>
4.7 Osteoblast Proliferation

4.7.1 Non Piezoelectric Treatment

Figure 20 shows the effect of ZnO/PVDF scaffolds (without piezoelectric stimulation) on osteoblast proliferation from 1 to 3 days. Osteoblast density on all groups increased from day 1 to day 3. However, compared with the control group, the scaffolds fabricated with 2 mg/ml ZnO NPs decreased cell density. These results indicated that osteoblast proliferation was not affected by ZnO/PVDF scaffolds when the ZnO NP concentration was below 1 mg/ml without piezoelectric stimulation, thus, 1 mg/ml was selected as the optimal ZnO NP concentration in this research.

![Graph showing osteoblast proliferation](image)

**Figure 20.** Osteoblasts density cultured on ZnO/PVDF scaffolds for 1 to 3 days, Values are mean ± STDEV; N=3. *P<0.05

4.7.2 Piezoelectric Treatment

The effect of 1 mg/ml ZnO/PVDF scaffolds (with piezoelectric treatment) on osteoblast proliferation from 1 to 3 days is shown on Figure 21. (a). Compared to the control group, ZnO/PVDF scaffolds significantly improved osteoblast cell density.
after day 1 and day 3, in which osteoblast density was 40% higher after 1 day of culture and 58% higher after 3 days of culture. The results indicated that osteoblast proliferation was stimulated by the piezoelectricity generated by the ZnO/PVDF scaffolds. Osteoblast cell density on all groups increased from day 1 to day 3, as shown in Figure 21 (b). All ZnO/PVDF groups had higher osteoblast cell density than the control group. However, when compared to the ZnO/PVDF scaffolds at different ZnO concentrations, the osteoblast densities after 3 days of culture on 0 mg/ml, 0.5 mg/ml and 1 mg/ml ZnO scaffolds were 38573 cells/cm$^2$, 37178 cells/cm$^2$ and 34976 cells/cm$^2$, respectively, which indicated that osteoblast density was slightly reduced when ZnO was added.

![Figure 21 (a). The effect of piezoelectric treatment on osteoblast (OB) density after 1 to 3 days of culture. Values are mean ± STDEV; N=3. *P<0.05](image)
Figure 21 (b). Osteoblast (OB) density cultured on ZnO/PVDF scaffolds (with piezoelectric treatment) for 1 to 3 days. Values are mean ± STDEV; N=3. *P<0.05

4.8 Bacteria Density

4.8.1 Without Piezoelectric Treatment

The effect of ZnO/PVDF scaffolds (with different ZnO concentrations) on SA, MRSA and E. coli 24 h growth are shown in Figure 22 (a), (b) and (c). Compared to the control sample, SA density on ZnO/PVDF scaffolds was inhibited from 38% to 64%, when ZnO concentration increased from 0.5 mg/ml to 2 g/ml. For MRSA, the rate of inhibition for 0.5, 1 and 2 mg/ml ZnO/PVDF scaffolds were 21%, 46% and 54%, in Figure 22 (b), respectively. For E. coli in Figure 22 (c), the density reduced 27%, 36% and 57%, when ZnO concentration was 0.5, 1, 2 mg/ml. The results indicated that the addition of ZnO NPs into PVDF scaffolds endowed the scaffolds with antibacterial properties. The antimicrobial ability also increased with greater ZnO NP ratios.
Figure 22. Colony counts for (a) *Staphylococcus aureus* (SA), (b) *Methicillin-resistant Staphylococcus aureus* (MRSA), (c) *Escherichia coli* (*E. coli*), Values are mean ± STDEV; N=3. (* P<0.05)
4.8.2 With Piezoelectric Treatment

The effects of 1 mg/ml ZnO in PVDF scaffolds (with and without piezoelectric treatment) on bacteria growth after 6 h are shown in Figure 23. Compared to the control group (no ZnO/PVDF scaffold, without piezo-excitement), ZnO/PVDF scaffolds (both with and without piezo-excitement) significantly decreased bacteria colonies after 6 hrs. The results indicated that 1 mg/ml ZnO in PVDF scaffolds inhibited bacterial growth, reducing SA, MRSA and E. coli density by 45%, 48%, and 37%, respectively. However, compared to the control (without scaffolds and piezo-excitation), groups without scaffolds but with excitation, had a lower bacteria density (29%, 32%, and 13% reduction in SA, MRSA and E. coli density), suggesting for the first time that piezo-excitation can reduce bacteria growth. Moreover, compared to the control group, groups with scaffolds and piezo-excitation had much lower bacteria numbers after 6 hrs (SA, MRSA and E. coli reduced by 68%, 70%, and 57%, respectively).

![Figure 23](image)

Figure 23. SA, MRSA, and E. coli colonies on the different scaffolds and treatments after 6 hrs. (Control group (-): No ZnO/PVDF scaffold without piezo-excitement, Control group (+): 1 mg/ml ZnO/PVDF scaffold without piezo-excitement). Values are mean ± STDEV; N=3. *P<0.05.
4.9 Correlation factor $R^2$ Calculation

In order to define the correlation of piezoelectric properties of ZnO/PVDF scaffolds with osteoblast density and bacterial forming units. The R Square and Adjusted R Squaue were calculated by Excel 2015. The result, as shown in Table 4 (all $R^2$ are beyond 0.98), suggest that piezoelectric properties are high related to osteoblast density and bacterial forming unit.

Table 5. Correlation Factors of Piezoelectric Properties with Osteoblast Density and Bacterial Forming Units (CFU)

<table>
<thead>
<tr>
<th>Correlation Factor</th>
<th>Osteoblast Density</th>
<th>SA CFU</th>
<th>MRSA CFU</th>
<th>E. coli CFU</th>
</tr>
</thead>
<tbody>
<tr>
<td>$R^2$</td>
<td>0.990</td>
<td>0.996</td>
<td>0.987</td>
<td>0.987</td>
</tr>
<tr>
<td>Adjusted $R^2$</td>
<td>0.985</td>
<td>0.994</td>
<td>0.981</td>
<td>0.982</td>
</tr>
</tbody>
</table>
5.0 Discussion

This study elucidated that ZnO nanoparticles can help to improve diverse qualities of electrospun ZnO/PVDF scaffolds, such as piezoelectricity, molecular structure, morphology and mechanical properties. Most importantly, these novel materials can increase osteoblast density and decrease bacteria colony forming units which are both badly needed for the future of orthopedic applications.

5.1 Fiber quality and morphology

During electrospinning, the applied voltage, solvent choice, and concentration of polymer solution have effects on fiber morphology and size. According to earlier studies [32,36,37], acetone and DMF were used as solvents in this experiment, a low concentration of PVDF (18%) was used to obtain the pure scaffolds with bead structures. The SEM image showed that by increasing ZnO concentration in the PVDF solution (from 0 to 2 mg/ml), the fibrous beads reduced significantly. The results suggested that adding ZnO NPs into the electrospun solution increased the conductivity and charge density of the scaffolds. Increased charge density of the polymer solution could cause a greater repulsion and a greater bending instability during electrospinning, which caused stretching of the fibers, resulting in a smaller diameter and reducing the total number of beads.

5.2 Fraction of β phase PVDF

In this study, to demonstrate the effects of ZnO NPs on the ratio of the β phase in PVDF scaffolds, the applied voltage was chosen as the middle voltage (15 kV) to avoid an extremely high and low β phase PVDF ratio. Moreover, when the
concentration of ZnO NPs increased from 0 to 2 mg/ml, the percentage of PVDF β phase increased by >10%. Compared to a previous study, Damarju et al. showed that by increasing the applied voltage from 15 kV to 25 kV, the β phase of PVDF improved to about 5% [36]. It is believed that the increased charging density was able to affect the β phase ratio. Previous research showed that by adding conductive materials, one can increase the conductivity of the electrospun solution and increase the charging density of the electrospun solution during electrospinning [65, 66]. Owing that the β phase PVDF can be formed in a strict electrical situation [34] (always over 10 kV), increasing the charging density assisted crystal phase changes raised the β phase percentage, which matched previous studies [66, 67].

5.3 Mechanical Properties

Interestingly, the mechanical test results showed that ZnO NPs at a 1 mg/ml concentration was optimal for the best Young’s modulus at 109.2 ± 7.9 MPa, maximum load at 4.802 ± 0.42 kN, and elongation at 140.16 ± 4.11%. The results indicated that the change in mechanical properties did not trend with ZnO concentration. The potential for the ZnO/PVDF scaffolds to be a ligament implant was shown for its high elongation and modulus (for example, the elongation at break and modulus of collateral human ankle joint ligament is about 15-25% and 90-150 MPa, so, the ZnO/PVDF scaffolds with 1 mg/ml ZnO NPs can be considered as a candidate for ligament replacement). A previous study showed that ZnO nanoparticles could enhance polymer composite strength but not flexibility [60], which was different in this research. Thus, the change of mechanical properties could be attributed to several factors, including morphology of fibers, PVDF β phase ratio, and ZnO concentration. Take bead density as an example, when the ZnO concentration was 0,
there was a high density of bead structures on the scaffolds which reduced the elongation of the scaffolds by causing stress discontinuity and putting more force on the fiber structure. However, when ZnO NPs concentration increased to 1 mg/ml, bead density decreased by over 80%, and bead influence was minimized. The result showed by adding ZnO NPs into an electrospun solution from 0 to 1 and 2 mg/ml, the bead density on the scaffold reduced significantly, from 1761 to 432 and 32 beads/mm². Different ZnO NPs concentrations and PVDF β phase fractions can also influence the mechanical properties of the fibers.

5.4 Contact Angle Analysis

The contact angles of ZnO/PVDF scaffolds were tested to evaluate the surface energy and its affect on osteoblast behavior and antibacterial ability of scaffolds. However, when the concentration of ZnO NPs increased from 0 to 2 mg/ml, the contact angle of scaffold surface was approximately 126° for all concentrations. Because scaffolds were fabricated by same working voltage, ZnO/PVDF scaffolds due have piezoelectric properties. However, when scaffolds were standing (without stimulation), piezoelectricity and charges were not generated. In addition, the weight ratio of ZnO/PVDF is under 1:50, so the low fraction of ZnO minimized the influence of ZnO NPs on surface energy of scaffolds. Then surface energy was mainly determined by PVDF. The result suggested that the surface energy of scaffolds did not change significantly, and therefore, it is not an important factor to cell behavior and antibacterial abilities in this research.
5.5 Piezoelectricity Detection

The piezoelectricity of ZnO/PVDF scaffolds at different ZnO concentrations was detected and estimated by electric meter in this study. The electric response from the ZnO/PVDF scaffolds at all ZnO concentration (ranging from 0 to 2 mg/ml) showed that ZnO/PVDF scaffolds do have the piezoelectric properties. Thus, it is believed that piezoelectricity occurred in the cell proliferation test and antibacterial test when piezo-excited. Moreover, the estimated electric responses showed the high ratio ZnO scaffolds due have better piezoelectric properties, which may cause by higher $\beta$ phase PVDF ratio and higher conductivity (both due to the higher ZnO NPs concentration).

5.6 Cell Proliferation

Bone is a kind of piezoelectric tissue with a piezoelectric coefficient $d_{15} = 0.1 - 0.3$ pC/N, and it has been shown that piezoelectric materials can enhance osteoblast proliferation and adhesion \cite{68}. In our test without piezo-excitation, when ZnO concentration reached 2 mg/ml, the osteoblast density after 3 days was 19% lower than the control group. The density of osteoblasts also decreased slightly in other groups which contained different concentrations of ZnO. However, results were quite different when the scaffolds were piezoelectric-excited. In the piezo-excited test, osteoblast cell densities on all samples were significantly higher than the control group. Take 1 mg/ml ZnO NPs group as an example, the osteoblast density was 20% higher than the control group after 3 days of cell culture. When compared to the non-piezo-excited 1 mg/ml group, the 1 mg/ml ZnO piezo-excited group even had a 60% higher cell density after 3 days of culture. This cell proliferation test result suggested that piezoelectric materials can promote osteoblast proliferation through
piezoelectricity, as the piezoelectricity may act as bio-electricity to stimulate bio-
reactions, which matched previous studies as we mentioned in section 2.4.1.

5.7 Antibacterial Ability

The antibacterial study indicated that ZnO NPs could provide antibacterial properties
to scaffolds, even without piezoelectric treatment. When ZnO NP concentration in the
scaffolds was 1 mg/ml, the density of SA, MASR and E. coli reduced by 45%, 48%,
and 37%, respectively, without piezoelectric treatment. When bacteria were subjected
to piezoelectric stimulation (also for 1 mg/ml ZnO scaffolds), the density of SA,
MASR and E. coli reduced by more than 68%, 70%, and 57%, respectively. The
reason why piezoelectricity can help inhibit bacteria growth may be the following
reason. When piezo-excited, the two sides of the scaffold were fulfilled with charges,
then one side become cationic and one side anionic. Because almost bacteria have
negative charges on their surface due to the proteins, acids and lipids that compose
their membranes, it is believed that the cationic scaffold surface could attract bacteria,
let bacteria close to the scaffold surface, then interact with a high concentration ZnO
NPs (because near to the scaffold) and killed by ZnO NPs. Additionally, according to
the previous study [69], anionic surface could slow down the initial adherent rate of
bacteria but would not inhibit bacteria growth after adhesion. Also, according to
piezoelectric coefficient of materials, it is possible to control which side is cationic or
anionic by modifying the deform shape of material. Thus, it is neccessary to let the
anionic surface attach to the bottom and cationic surface towards to medium and
bacteria (to get a better antimicrobial ability). Moreover, the results from piezoelectric
excitation tests without piezoelectric scaffolds also showed that the stimulation
method (dynamic stretching) can lead to silicone well plate bottom movement,
inhibiting bacterial attachment to the surface, making it difficult to form a biofilm.
This dynamic stimulation method can also accelerate ZnO NPs diffusion, which can help to inhibit bacterial growth. Thus, there are at least three factors that can inhibit bacterial growth in the present piezo-excited test: piezoelectricity, dynamic stimulation, and ZnO diffusion. Future studies will be needed to elucidate the role of each factor on reducing bacteria growth. None-the-less, this is the first study to the best of the authors’ knowledge that shows reduced bacteria growth on piezoelectric materials when piezoelectric-stimulated (without using antibiotics).
6.0 Conclusions

A novel kind of piezoelectric orthopedic scaffold has been investigated in this thesis. By adding ZnO NPs into PVDF scaffolds, the ratio of β phase PVDF, mechanical properties, scaffold morphology, osteoblast proliferation, and bacteria density changed significantly. The percentage of β phase PVDF was 10% higher than 0 mg/ml ZnO scaffolds when ZnO content in the scaffolds was 2 mg/ml. PVDF scaffolds during electrospinning can also enhance mechanical properties of scaffolds, when ZnO concentrations reached 1 mg/ml, the Young’s modulus, max load and elongation increased significantly compared to the pure PVDF scaffolds. In summary, by using the new discoveries in this study, one can find an optimal ZnO concentration and fabricate a ZnO/PVDF scaffold with a high ratio of β phase PVDF and strong mechanical properties which can stimulate osteoblast proliferation and inhibit bacterial growth under piezo-excitation.

Moreover, these new discoveries indicate that ZnO/PVDF scaffolds can be used in the orthopedic field, like as a bandage or replacement to help heal an injured ligament. They are also applicable in other fields that require better mechanical and electrical properties, like the electronic device field. Future studies are needed to measure the piezoelectric coefficient of ZnO/PVDF with a different β phase PVDF ratio, the conductivity coefficients, and the piezoelectric effect of the scaffolds on other functions of osteoblasts (e.g. protein adsorption and calcium deposition). In addition, further tests need to be completed using an electrical supply to imitate piezoelectricity and elucidate the piezoelectricity antibacterial effect from other factors.
7.0 RECOMMENDATIONS

7.1 Clarification of Piezoelectric Effects on Bacteria Growth

Although this thesis provides evidence that piezoelectric effects can stimulate osteoblast cell proliferation and inhibit bacteria growth, additional studies to clarify the precise effect of ZnO/PVDF scaffolds on bacteria growth are necessary. Piezoelectricity can form an alternative current (AC), because charges were generated by the deformation and reformation of piezoelectric materials [70]. Therefore, we can use micro-current (AC) to imitate piezoelectricity. An oscilloscope is needed to monitor the accurate piezoelectricity of ZnO/PVDF [71]. When piezoelectricity was measured, an alternative current (same voltage and amps to piezoelectricity) can be used to treat the bacteria.

7.2 Electrospinning Operation Parameter Optimization

In this thesis, electrospinning operation parameters included a 15 KV working voltage, a 16.5 cm working distance and a 2 mg/ml electrospun solution flow rate. However, these parameters are not optimal parameters to obtain scaffolds of the best quality. From a previous study, the working voltage distance and flow rate can also influence fiber quality [72]. Thus, it is necessary to optimize operating parameters and obtain scaffolds of better quality for future study.

7.3 Alternatives ZnO NPs Additives

Although ZnO NPs were approved as potential additives to improve the morphology, enhance mechanical properties, and change the crystallinity of PVDF scaffolds during electrospinning, it is still necessary to find alternative NPs that can be used in this area. It is not for sure if ZnO NPs are the best additives for PVDF electrospun fibers or not.
Also, whether ZnO NPs can improve other polymeric electrospun fibers in the same way (crystallinity, mechanical properties and morphologies) is not clear. There are several other additives that have been used as electrospun additives. For examples, Ag NPs [773] and TiO₂ NPs [74] have also been investigated for their good antibacterial abilities. Additionally, Ag is well known for its good conductivity [75] and TiO₂ [76] is famous for optimal mechanical properties, which suggests that these additives could better replace materials to improve diverse characteristics of PVDF scaffolds.

7.4 The Effect of ZnO/PVDF Scaffolds on Other Cells

Although the effects of the ZnO/PVDF scaffold (with and without piezoelectric excitation) on osteoblasts were investigated, the investigation on other types of cells are also needed. The first aim of this study is to discover a material that can help ligaments recover from injury and even replace parts of ligament in human bodies. The data from osteoblast is not enough, because osteoblast is only one kind of cell that ligaments contain. Fibroblasts are another important cell that interact with ligaments and form the ligament-to-bone interface [77]. In a previous study, fibroblasts have been stimulated by piezoelectric materials (PU/PVDF). Therefore, it is better to test fibroblast with piezoelectric excitation, and examine whether ZnO/PVDF scaffolds could stimulate fibroblast proliferation or not.

7.5 Quantify Piezoelectric Coefficients of ZnO/PVDF Scaffolds

Although piezoelectricity was detected in this research, it is still qualitative measured. The affect of different concentrations of ZnO NPs on piezoelectric properties was not quantified. Therefore, it is necessary to test the piezoelectric properties of scaffolds quantitatively. Several constants can quantify piezoelectricity and the piezoelectric
coefficient $d_{33}$ is the most commonly used coefficient for PVDF materials $^{78}$. Thus, it is necessary to test $d_{33}$ coefficient by piezoresponse force microscopy (PFM) $^{79}$ in the future.
# 8.0 NOMENCLATURE

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BaTiO₃</td>
<td>Barium Titanate</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DI Water</td>
<td>Deionized Water</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic Light Scattering</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
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<tr>
<td>FTIR</td>
<td>Fourier Transform Infrared Microscopy</td>
</tr>
<tr>
<td>HA</td>
<td>Hydroxyapatite</td>
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<tr>
<td>MRSA</td>
<td>Methicillin-resistant Staphylococcus aureus</td>
</tr>
<tr>
<td>MTS</td>
<td>Proliferation Assay Kit (Colorimetric)</td>
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<tr>
<td>MW</td>
<td>Molecular weight</td>
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<tr>
<td>NPs</td>
<td>Nanoparticles</td>
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<tr>
<td>OB</td>
<td>Osteoblast</td>
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<tr>
<td>OD</td>
<td>Optical density of absorbance</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PFM</td>
<td>Piezoresponse Force Microscopy</td>
</tr>
<tr>
<td>PEO</td>
<td>Poly(ethylene oxide)</td>
</tr>
<tr>
<td>PLLA</td>
<td>Poly(lactic acid)</td>
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<td>PS</td>
<td>Polystyrene</td>
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<tr>
<td>PU</td>
<td>Polyurethane</td>
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<tr>
<td>SA</td>
<td>Staphylococcus aureus</td>
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<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
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<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
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<thead>
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<tr>
<td>TSB</td>
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<td>UV</td>
<td>Ultra Violet</td>
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<td>ZnO</td>
<td>Zinc Oxide</td>
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<tr>
<td>3D</td>
<td>Three Dimensional</td>
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</tbody>
</table>
9.0 References


19. Lhoste K. *Development of PVDF micro and nanostructures for cell culture studies*, Université René Descartes-Paris V; 2012.


69. Gottenbos, B., Grijpma, D. W., van der Mei, H. C., Feijen, J., & Busscher, H. J.


