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Three-Dimensional Hybrid Piezoelectric Polymer-Based Scaffolds for Regenerative Medicine and Biosensors

A Thesis submitted in partial satisfaction
of the requirements for the degree of

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in

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by

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ABSTRACT OF THE THESIS

Three-Dimensional Hybrid Piezoelectric Polymer-Based Scaffolds for Regenerative Medicine and Biosensors

by

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Dr. Huinan Liu, Chairperson

Electrical and ultrasonic stimulation have both shown great potential for promoting cell proliferation and tissue repair since piezoelectric materials can produce ultrasound with electrical stimulation and produce electrical signals with ultrasound stimulation. They can be a great option for tissue regeneration and biosensors. To evaluate the possibility of Polyhydroxybutyrate(PHB), a piezoelectric material to be used for medical use, cell experiments are carried out without any stimulation applied to the materials first to study their effects on cells. Results show that the materials do not affect the density of cells while altering the cells' morphology which is related to the materials' fiber structures. Since the material is cell compatible, further experiments with 3D-printing scaffolds and ultrasound stimulation can be carried out to test the effect of ultrasound and electrical stimulation on cells grown on the materials. In this thesis, we printed 6 different material groups including pure PHB scaffolds, PHB scaffolds modified with different percentages of either polyaniline (PANi) or reduced graphene oxide (rGO) to vary the materials' piezoelectric

properties. Future work will focus on using ultrasound to remotely stimulate 3-D printed Polyhydroxybutyrate(PHB) scaffolds and test their effects on cells' proliferation.

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Chapter 1 Introduction and Background

Focusing on piezoelectric materials, electrospun piezoelectric fibers offers excellent flexibility and improved strength, so it is expected their exploitation in a wide variety of applications. Various cell types (including neurons,neural stem cells, and fibroblasts) have been successfully stimulated by taking advantage of particles or fibers showing piezoelectric behavior. There are a few strategies already applied, like implantation of autografts, allografts and xenografts, providing grafts for the patient, cadavers and animals. But using the autograft repair system has some major disadvantages like loss of function at the graft site, mismatch of damaged cell and graft dimensions.

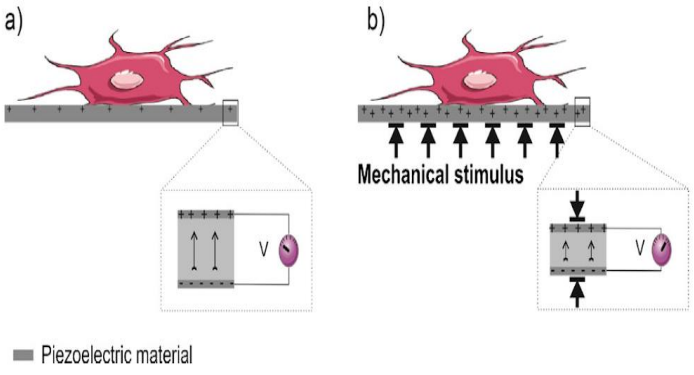

Figure1. Mechanisam of piezoelctric materials.

On the other hand, allogeneic and xenogeneic tissues have the advantages of their availability, along with the benefit of not requiring harvesting from patients. However, their disadvantages including disease transmission and problems of immunogenicity need to be considered seriously.[10] Tissue engineering or regenerative medicine offers a promising approach to repair damaged tissues by combining cells with bio-materials that

act as scaffolds to facilitate tissue growth. The Biomaterial can be designed to mimic the native tissue extracellular matrix providing appropriate cues for desired cell function.

Piezoelectric effect is not a completely novel idea since it has been known since the 19th century with broad technological applications since the beginning of the 20th century. [1] The discovery of piezoelectric effect is connected with the names of famous physicists Jacques Curie and Pierre Curie who recognized that anisotropic crystals i.e. crystals without center of symmetry can generate electric dipole when mechanically squeezed. The electric dipole is also called piezoelectricity. The described effect can work in oppose way when an anisotropic crystal become deformed due to voltage imposed on it. The aforementioned phenomenon is depicted. The mechanical deformation is , however, a simple situation and oscillation is rather chosen in the common applications like here described analytical devices. [2] In the case of oscillation, an alternating voltage is imposed on the crystal and mechanical oscillation then occurs.

1.1Poly(3-hydroxybutyrate)

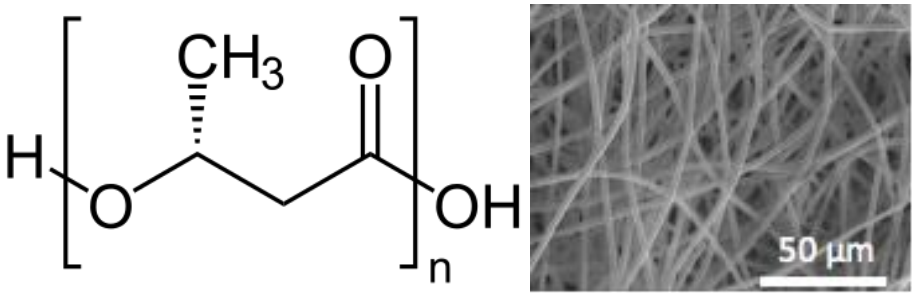

Figure 2. Chemical structure of Poly(3-hydroxybutyrate)

Polyhydroxybutyrate (PHB) is a polyhydroxyalkanoate (PHA), a polymer belonging to the polyesters class that are of interest as bio-derived and biodegradable plastics. The

poly-3-hydroxybutyrate (PHB) form of PHB is probably the most common type of polyhydroxyalkanoate, but other polymers of this class are produced by a variety of organisms: these include poly-4-hydroxybutyrate (P4HB), polyhydroxyvalerate (PHV), polyhydroxyhexanoate (PHH), polyhydroxyoctanoate (PHO) and their copolymers.[3] PHB is produced by microorganisms (such as Cupriavidus necator, Methylobacterium rhodesianum or Bacillus megaterium) apparently in response to conditions of physiological stress; mainly conditions in which nutrients are limited. The polymer is primarily a product of carbon assimilation (from glucose or starch) and is employed by microorganisms as a form of energy storage molecule to be metabolized when other common energy sources are not available. Microbial biosynthesis of PHB starts with the condensation of two molecules of acetyl-CoA to give acetoacetyl-CoA which is subsequently reduced to hydroxybutyryl-CoA. This latter compound is then used as a monomer to polymerize PHB. PHAs granules are then recovered by disrupting the cells.[4]

Poly(3-hydroxybutyrate) (PHB), and copolymers of PHB with 3-hydroxyvalerate (P(HB/HV)), are produced by certain bacteria. The fiber structure of PHB and P(HB/HV) is mainly composed of α-form crystals of PHB, but small amounts of β-form crystals are known to coexist. The molecular chain in the α-form crystal is in 3_1 helix conformation, whereas that in the β-form crystal is almost in the planar zigzag conformation (Orts et al. 1990). The α- and β-form crystals usually exhibit ordinary c-axis orientation, and the amount of β form varies depending on the drawing and annealing conditions (Furuhashi

et al. 1998) or the take-up velocity in the high-speed spinning process (Schmack et al. 1999)[6].

In some cases, the α-form crystals in drawn and annealed fibers exhibit peculiar bimodal orientation, which is represented by the coexistence of diffraction spots and rings in the WAXS pattern. Some rings are complete, with a higher intensity near the meridian, while other rings are incomplete, with a lack of intensity near the meridian. Detailed analyses of the ring pattern reveal that the c-axis is preferentially oriented perpendicular to the fiber axis, whereas the a- and b-axes are randomly distributed around the c-axis (Furuhashi et al. 1998).

1.2 Polyaniline

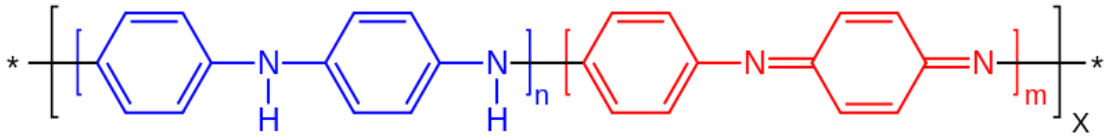

Figure 3 Chemical structure of Polyaniline

The aera of intrinsically conducting polymers has started with the invention of polyacetylene in the year of 1958. However, polyaniline, which is commonly abbreviated as PANI or PAni, has attracted much more attention of the researchers because it is a cheaper monomer compared to polyacetylene and its ease of synthesis. Although the research of Alan G. McDiarmid, Hideki Shirakawa, and Alan J. Heeger is considered as the pioneering work in the field of conducting polymer, for which the Nobel prize was conferred to them in the year of 2000, the history of PANI is even much older than polyacetylene and many other conducting polymers. In 1862, Dr. Henry Letheby, a

professor in the College of the London Hospital, has reported the first ever synthesis of "a blue substance" during electrolysis of aniline sulfate (AS), which decolorizes partially while treated with reducing agent [7] In the early ages, it was termed as "aniline black" because of its dark pigment color, which was used for dyeing of textile fabrics. Since then, intensive research in this field explored its potential applicability in diversified fields. Doping with acids enhances improves electroactive behavior, whereas dedoping of doped PANI in presence of base deteriorates its electroactive characteristics. Doping/dedoping-based tuneable wide range of electrical and electrochemical properties of PANI as well as wide varieties of nano-/microstructures facilitate its applicability in these areas. Reversible redox behavior of PANI is useful for supercapacitor, gas sensor, pH sensor, fuel cell applications. Electromagnetic interference (EMI) shielding application involves frequency-dependent conductivity of PANI. Moreover, structures of PANI can also be tuned within a wide domain of shape, size, and crystal structures.

1.3 Reduced Graphene Oxide

The preparation of reduced graphene oxide by chemical reduction of graphene oxide usually involves highly toxic reducing agents which are harmful to the environment and human health. A mediated facile and relative green approach for the preparation of reduced graphene oxide in ethanol using artemisinin as a reducing agent was reported. Figures 3.38a and b display typical transparent morphology and rippled surface of graphene oxide and reduced graphene oxide, respectively.

The diffraction spots are ring-like partially destroyed during oxidation-reduction. The typical thickness of the reduced graphene oxide platelet is about 1.2 nm, corresponding to 3 layers and interlayer distance is 0.34 nm typical of the natural graphite.

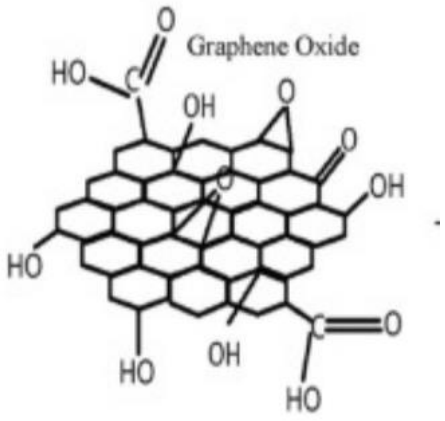

Figure 4. Chemical structure of Reduced Graphene Oxide

After reduction by artemisinin, reduced graphene oxide layers are comprised of defect-free nanosize graphene-like domains surrounded by the topological defect areas dominated by pentagons-heptagons pairs or quasi-amorphous.

1.4 Polycaprolactone

Polycaprolactone is another biodegradable polyester (Chu 1995, Saad et al. 1999, Yoda 1998). The ring-opening polymerization of ϵ-caprolactone yields a semi-crystalline, hydrophobic polymer with a melting point of 63 °C and a glass-transition temperature of −60 °C. Because the homopolymer has a degradation time on the order of two years, copolymers have been synthesized to accelerate the rate of bioabsorption. For example, copolymers of ϵ-caprolactone with dl-lactide have yielded more flexible materials with

higher degradation rates than polycaprolactone. The high permeability of polycaprolactone to various agents has made it an important candidate for the development of drug delivery systems.

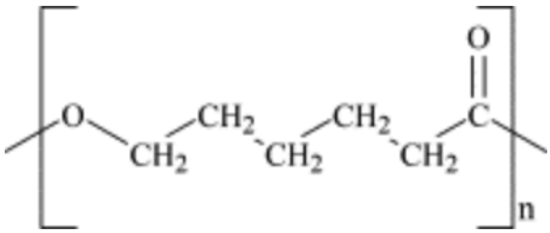

Figure5 Chemical structure of Polycaprolactone

Polycaprolactone is a bioresorbable semicrystalline poly(α-hydroxyester). It degrades slowly by hydrolysis due to its high crystallinity and hydrophobic nature. It was used in many fields, such as implantable biomaterials, biodegradable materials, and microparticles for drug delivery [18,19]. The new descent of biomaterials is going to be involved and active, therefore competent at smooth linkage with encirclement tissues. Particularly, it is needed for materials, which can integrate stimulating cues.

1.5 3D Printing

3D printing is another method for producing the tissue scaffolds with better control of pore size, complex shapes, size and porosity when compared with other fabrication technologies. 3D printing works on the principle of laying down of successive layers of the polymer to form the 3D scaffold. There are different methods in 3D printing available such as fused deposition modelling, inkjet printing, and selective laser sintering, colorjet printing and steriolithography. 3D printing technology has been tried to produce different scaffolds for trachea [, bone, oesophagus and aortic valve.

Advances in 3D printing have led to bioprinting recently. Bioprinting is a 3D printing technology using living cells with the preservation of the viability of the cells within the scaffold. One of the fully functional human tissue that was bioprinted is Organovo's exVive3D Liver that has been used to provide toxicity assessment that is supplementing in vitro and preclinical animal testing.

Figure6.3D Bioplotter

3D printing is a process to produce objects by adding selected materials together where necessary following digital 3D model guidance. The significances of this technology, such as the ability to construct complex geometric structures matched to patient's anatomy or surgeon's requirement, enable its wide applications in medicine, ranging from surgical planning tools to custom surgical devices. The translation of 3D printing in health care has been enhanced recently under the guidance of the regulatory agency. The focus of the present work is to demonstrate the translation aspects of 3D

printing in medical devices. An overview of current 3D printing technologies, regulatory guidance, and 3D printed medical devices in the market is carried out in this chapter, along with a brief introduction of laboratory concepts such as bioprinting and 4D printing.

1.6 Cell Culture Experiment

Cell culture is the technique in which cells are removed from an organism and placed in a fluid medium. Under proper conditions, the cells can live and even grow. The growth can be characterized by cell division (mitosis) or by other processes, such as differentiation, during which the cells can change into specific types that are capable of functions analogous to tissues or organs in the whole organism. The practice of cell culture (and its close cousins, tissue culture and organ culture) originated in a Yale University laboratory in 1907, when Ross Harrison removed nerves of a frog and maintained them in a simple salt solution for several days. Within a very few years a visiting scientist in Harrison's laboratory, Richard Goldschmidt, reported on the first cell cultures from an insect. For the next half-century, insect cell culture was used periodically in a variety of experiments, such as studying the pathogenesis of viruses, but the field received a great boost when the Australian Thomas D. C. Grace succeeded in obtaining four cell lines from the emperor gum moth, Antheraea eucalypti. These lines were capable of continuous growth, requiring periodic subculturing.

1.7 References

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Chapter 2 3D-Printing Piezoelectric Polymer-based Scaffolds

2.1 Introduction and previous studies

Piezoelectric materials are now under active research and are widely used in different applications. Recent years, more and more researches are focusing on the possibility to apply their piezo electric properties for biomedical uses [1]. In this research, our team uses 3D-Bioplotter to remotely 3-D printed Polyhydroxybutyrate(PHB), a biodegradable piezoelectric polymer, and test the effect of its effect on cells' proliferation. If the scaffolds are made of biodegradable materials such as PHB, the implants can even vanish by itself and do not need to be removed by another surgery which may lead to further risks for patients [2].

Poly(3-hydroxybutyrate) (PHB) are a class of polyesters that can be naturally produced by bacteria. Poly(3-hydroxybutyrate) (PHB) has attracted attention for application in biomedical areas as scaffolds due to its biocompatibility and biodegradability [3]. PHB is a thermoplastic polyester classified as biopolymer, which is produced by micro organ-isms, as the bacteria Ralstonia eutropha, under imbalanced growth conditions. The family of PHB biopolymers has been widely used for biomedical applications, such as bone plates, sutures, rivets, staples, screws, orthopedic pins, bone marrow scaffolds and meniscus re-generation devices [4].

In the previous studies[5], we can conclude that our materials do not show signal of cytotoxicity to BMSCs, thus, further studies can be carried out with 3D printing and external stimulation to utilize the materials' piezoelectric property to generate stimulation to desired locations for potential tissue repairing and regenerative medicine applications.

2.2 Materials and Methods

2.2.1Materials Preparation

Five different components of materials are used in the experiments including PHB, PHB 98% - 2% PANi (PHB-2%PANi), PHB 97% - 3% PANi (PHB-3%PANi), PHB 99.8% - 0.2% rGO (PHB-0.2%rGO) and PHB 99% - 1% rGO (PHB-1%rGO). PHB is the major component of the materials which can offer piezoelectricity, rGO and PANi are used to adjust the mechanical property and electrical conductivity of the materials. PHB is purchased from Goodfellow, Inc. It comes in granule with a nominal granule size of 5mm. The granule has a dielectric constant of 3.0 at 1MHz and volume resistivity of 10^16Ohm. The material also contains 4% polyhydroxyvalerate(PHV), a copolymer of PHB. PANi is purchase from Sigma-Aldrich and it has an average mol weight~100,000 and conductivity of 1 × 10^-9 S/cm. RGO is purchased from Graphenea,Inc. and it has conductivity of 6.67 S/cm and density of 0.06-0.09 g/mL.

First, the weight of PHB is measured and PHB is mixed with chloroform at 10% weight percentage concentration in a close-lid glass bottle. Second, the mixture is heated and stirred with water bath on a hot plate stirrer. The water bath is controlled at 60℃ and the stirring speed is 500rpm. Third, PANi and rGO particles are dispersed in chloroform and the two solutions are mixed together. Then the mixed solutions are ultrasonicated(5 mins, 5s on/off) and speedmixed (2500rpms, 3mins) for three cycles to ensure uniform distribution of the particles. Then the solution is cast into multiple 1mm thick sheets on a 100℃ hot plate to allow fast evaporation of chloroform. Next, the sheets are cut into small pieces and further dried in oven at 80℃ for 3 hours. Then the material is again

dissolved in chloroform with PHB at 15% weight percentage, again, 60℃ water bath and 500rpm stirring rate are used with the same hot plate stirrer. Finally, the lids are open and redundant chloroform is evaporated for 1-2 hours to reach suitable viscosity for 3-D printing of the materials.

5mL of the materials are injected into a 30mL syringe and a 0.6mm diameter needle is used for 3-D printing, a two-layer net pattern is used for printing, the size of the whole pattern is 15mm*15mm and the spaces between two neighboring lines are 1.5mm. After printing, each piece is cut into four 7.5mm*7.5mm pieces for cell experiments.

2.2.2 Materials Characterization

Different techniques are used for material characterization. SEM with EDS is used to study the morphology of the materials. XRD is used to study the crystal structure of the materials, FTIR is used to determine the functional groups in the materials. In addition, tensile test is used to study the mechanical properties of the materials.

The morphology of the samples was studied via scanning electron microscopy (SEM) using a scanning electron microscope (SEM; Nova NanoSEM 450, FEI Co., Hillsboro, OR, USA) equipped with an X-Max50 detector and AZtecEnergy software (Oxford Instruments, Abingdon, Oxfordshire, UK) before and after experiments with cells as well as energy dispersive X-ray spectroscopy (EDS) used to analyze the element compositions of the materials. The accelerating voltage is 5kV.

The thin sheet after casting is used for tensile test. The samples are cut into 35mm*8mm*0.2mm pieces, the middle part of the samples is narrower than the holding part to ensure the samples break in the middle. Instron 5969 Dual Column Testing

System with 5N max wedge action grips is used for testing. Stress and strain are calculated from the data gained from the test.

Bone marrow stromal cells (BMSCs) are collected from rats at age around 2 weeks, the cells are cultured 3 days before experiments and the cell numbers are counted with the hemocytometer. 10,000 cells per cm2 are seeded in each well with the materials at the bottom of the wells. The cells are cultured in incubator at 37 Celsius Degree and 5% CO2 level for 24 hours. During the culturing, the samples are stimulated for 20 seconds every 2 hours. Then the cells are fixed with 4% paraformaldehyde and stained with Alexa Flour 488(for cell skeleton) and DAPI(for cell nuclei).

2.3 Data analysis and discussion Material Preparation:

2.3.1 3D Printing and defect discussion

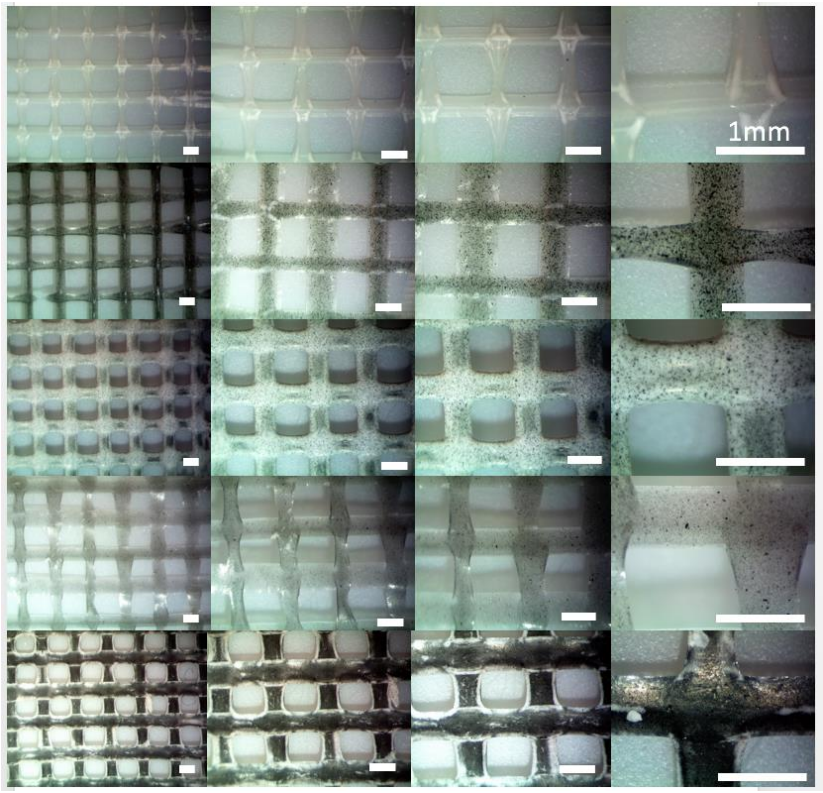

Image 7. Image of 3D printing scaffolds

The 3-D printed materials are shown in Figure7. The images are taken with Amscope, four different magnifications are shown in the images. Compared with the PHB powder we purchased from Sigma, the PHB we use in the experiments is very difficult to dissolve, which might be caused by the 4% PHV component in the PHB

2.3.2 SEM

The morphology of the samples was studied via scanning electron microscopy (SEM) using a scanning electron microscope (SEM; Nova NanoSEM 450, FEI Co., Hillsboro, OR, USA) equipped with an X-Max50 detector and AZtecEnergy software (Oxford Instruments, Abingdon, Oxfordshire, UK) before and after experiments with cells as well as energy dispersive X-ray spectroscopy (EDS) used to analyze the element compositions of the materials. The accelerating voltage is 5kV.

2.3.3 XRD

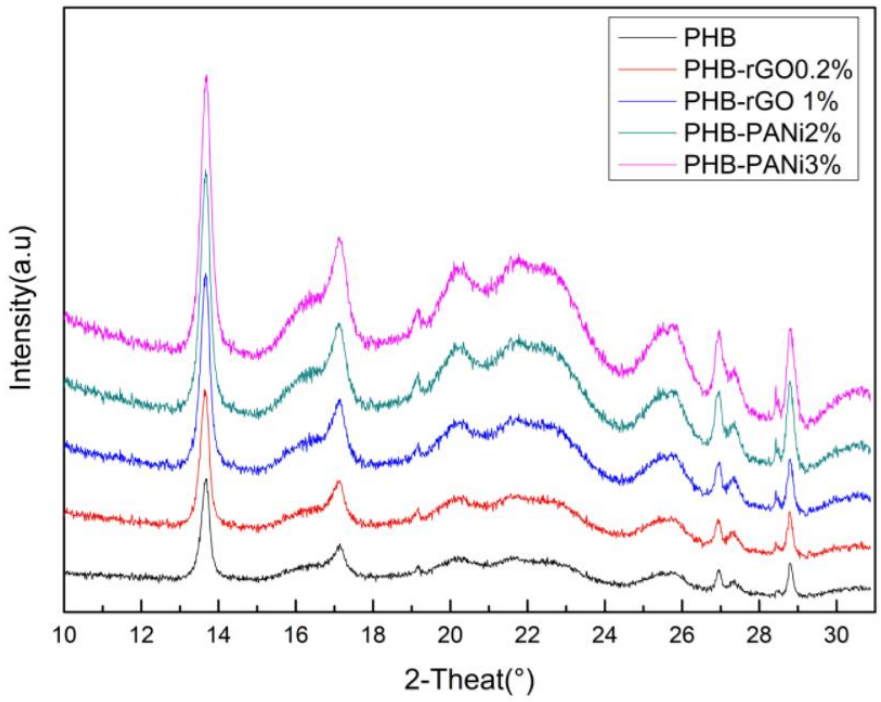

Figure8. XRD image of samples

The XRD result is shown in Fig8. We cannot observe any significant difference among the peaks, all five samples show peaks around 13.5°,17°, 27°and 29°, which means adding PANi and rGO particles do not alter the crystal structure of PHB.

2.3.4 FTIR

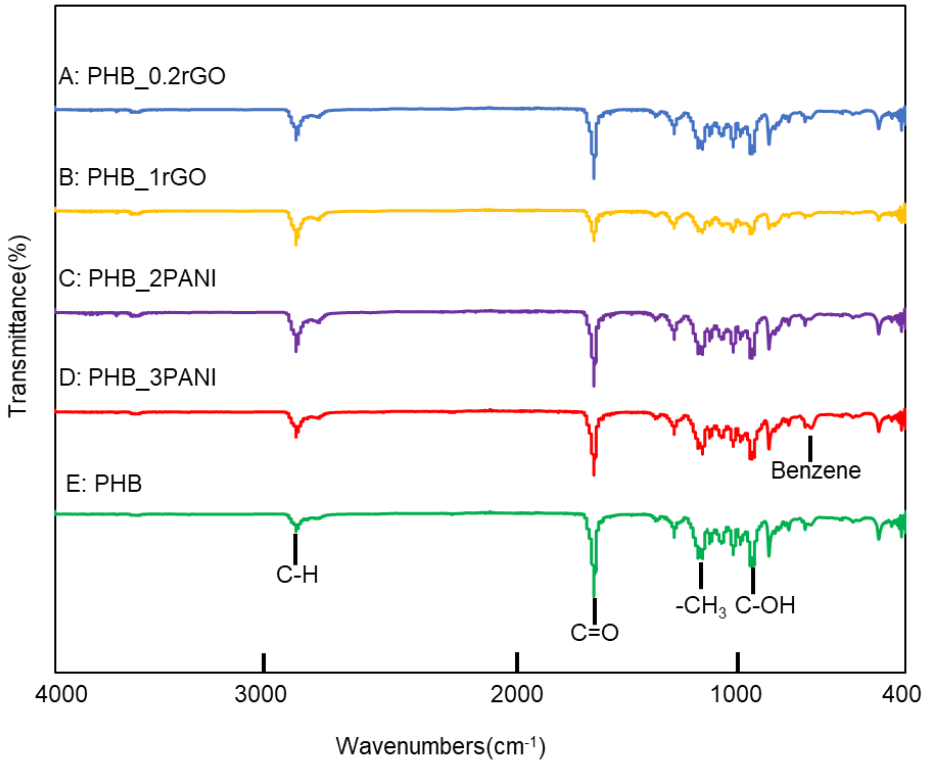

Figure 9. FTIR spectra of samples. (A) PHB_0.2rGO, (B) PHB_1rGO (C) PHB_2PANi, (D) PHB_3PANI, (E) PHB.

The FTIR result is shown in Fig9. It can be observed that the characteristic peak of PHB is obvious at around 1730nm(C = O) and 1279nm(C-O-C). There are multiple peaks at 1138-829nm range for C-O and C-C stretching vibration. Addition of PANi does not

alter the FTIR result since the percentage of PANi is quite low and the characteristic peak of PANi, Para-disub. Benzene, falls in 860-800nm range which overlaps with the peaks in PHB.

2.3.5 Tensile test

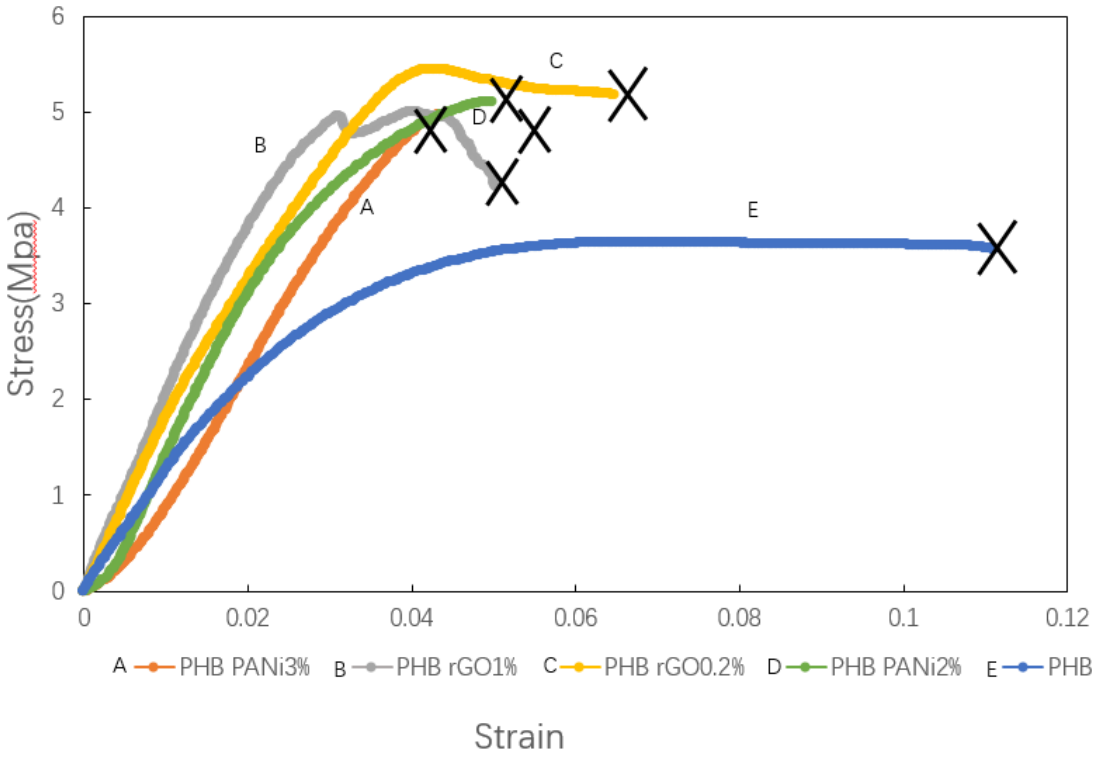

Image 10. Image of Tensile test for 5 samples

The tensile test result is shown in Fig10. We can observe that the addition of PANi and rGO particles improved the mechanical property of PHB, the ultimate strengths are higher in these materials. However, the fracture strain is shorter than pure PHB, which means the addition of the particles make the material more brittle. The thin sheet after casting is used for tensile test. The samples are cut into 35mm*8mm*0.2mm pieces, the middle part of the samples is narrower than the holding part to ensure the samples break

in the middle. Instron 5969 Dual Column Testing System with 5N max wedge action grips is used for testing. Stress and strain are calculated from the data gained from the test.

2.4 Potential Improvements and Future work directions

Problems such as brittleness, lack of specific cell-bind site are also challenges need to be solved before the materials can be put into widely use in biomedical field[43]. Since there are some differences between in vitro culture environment and in vivo, in vivo studies need to be carried out before the materials can be used for biomedical applications. Another important property of the materials is the piezoelectric property. In this study, we have shown that the piezoelectric properties change with the add-in either rGO or PANi, however, no external stimulation is applied in this study, in future studies, we will continue with the materials with external stimulation to change the surface charges of the materials and study their influence on cells.

2.5 Conclusion

From the previous studies, we can conclude that our materials do not show signal of cytotoxicity to BMSCs, thus, further studies can be carried out with external stimulation to utilize the materials' piezoelectric property to generate stimulation to desired locations for potential tissue repairing and regenerative medicine applications. In this experiment, we successfully printed the PHB based scaffolds. We also have done some analyze experiments. In next step, we will focus on Ultrasound Stimulation platform building up and in vitro cell stimulation test.

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