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Bioengineering

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Registration number: 190178806

Module code: IPE BIE

Module name: Bioengineering Final Year Projects

Assignment title: Final Year Project

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Date submitted: 26/05/2022

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Bioengineering

Final Year Project

Assessing the biocompatibility of Polyhyroxyalkanote polymers P(3HB) and P(3HO-co-3HD) in nervous tissue engineering applications

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May, 2022

Supervisors: Ipsita Roy and Emmanuel Asare

Dissertation submitted to the University of Sheffield in partial fulfilment of the requirements for the degree of Bachelor of Engineering

Acknowledgements:

I would like to thank my project supervisor Professor Ipsita Roy, for both her encouragement and contribution to the literature behind this project. I would also like to thank my mentor, Emmanuel Asare, for his teachings and helpfulness throughout the length of this project.

Thanks to David Gregory for taking the SEM images of the nerve guide conduits shown in this report. Many thanks to Annabelle Fricker for supervising the live/dead staining experiment and confocal microscopy imaging. And finally, I would like to give credit to my lab partner, Ayan Bartels-Ellis, for being part of this project and being there for me when I needed support.

Table of Contents

1 – Abstract

Nerve guidance is starting to be considered the potential successor of autologous nerve graft surgery in the treatment of peripheral nerve damage. This new medical technology has overcome many obstacles and is currently on the verge of a breakthrough. As more research is being put into nerve guidance, they are closer than ever to becoming a viable alternative to nerve autograft surgery, which has numerous disadvantages for the patient. In the present study, Poly(3-Hydroxybutyrate) (P(3HB)) as well as a poly(3-hydroxyoctanoate-co-3-hydroxydecanoate) (P(3HO-co-3HD)), were obtained through two separate controlled bacterial fermentation of *Bacillus subtilis* and *Pseudomonas mendocina* respectively. These polyesters were then extracted from the cellular walls of the bacterial species and purified through a two-stage Soxhlet extraction method. The purified polyesters were then tested for biocompatibility with NG108-15 neuronal cells through a live/dead analysis, a resazurin assay to quantify metabolic activity and a neurite extension measurement. After a positive confirmation of the biocompatibility of the polymers, a 50:50 blend of P(3HB) and P(3HO-co-3HD), as well as an 80:20 blend, were moulded into the shape of nerve guide conduits using a dip coating machine. The conduits were then imaged by SEM (scanning electron microscopy) to reveal the surface architecture of the conduits. Finally, nerve guide conduits made from a 50:50 blend of P(3HB) and P(3HO-co-3HD) were encapsulated with neurotrophic growth factors NGF (nerve growth factor) and BDNF (brain-derived neurotrophic factor) at different blends and concentrations. Unfortunately, the ELISA test (enzyme-linked immunosorbent assay) to quantify the rate of release of NGF and BDNF over time was not performed, therefore the rate of release of NGF and BDNF was not included in this report. This study promotes nerve guidance as an alternative to autologous nerve graft surgery, it also advocates for the use of polyhydroxyalkanoates polymer blend 20% P(3HB) and 80% P(3HO-co-3HD) as a scaffolding material for nervous tissue engineering and it encourages further experimentation with nerve guide conduits made from this polymer blend which release growth factors and/or contain supportive cells.

2 – Introduction

Biomedical engineering is a newly emerging field that combines knowledge of material science, engineering, and biology to design and manufacture medical devices whose application ranges from wound healing to total limb replacement. Biomedical engineers have constructed medical apparatus that can encapsulate drugs and safely deliver them to our system [1], they have designed implantable heart pacemakers that can control the beating rate of the heart to prevent life-threatening heart failures [2]. Biomedical engineers

have even made fully functional artificial limbs that have helped numerous disabled that have lost their limbs walk or use their hands again [3], [4]. Because of this innovation, the biomedical devices global market was worth 432.23 billion US dollars in 2020 and is expected to reach a total value of 657.98 billion dollars by the year 2028 [5]. The growth of this market can be attributed, in a large part, to the many engineers and scientists that have helped to advance the field of biomaterial science and consequently created, or discovered, the extracellular scaffolding materials that biomedical engineers needed to construct the biocompatible and biodegradable implantable medical devices that solved many of the medical conditions that our population has always been suffering from. As of today, a large amount of experimental research is being put into a new medical implant designed for nervous tissue engineering applications, the device is referred to as a nerve guide conduit or an NGC. This implantable device has the potential to greatly decrease the need for autograft surgery following consequent injury to a patient's peripheral nervous system [6], [7]. The standard nerve autograft procedure requires two separate surgical operations to be performed on the patient, the first one is to remove healthy nerve fibres from a designated donor site on the patient's body, and the second surgery involves the grafting of the neuronal cells onto the site of injury [8]. The downsides of this surgery are its high cost, on average costing 3,154 ±704 US dollars [9]. The invasive nature of the operation as it requires two separate surgeries, one to remove nerves from the donor site and another to graft them on the site of injury. And lastly, the loss of sensation on the patient's donor site following the surgery [10]. A nerve guide conduit on the other hand is inserted once at the site of injury and does not require any additional surgery to be removed after it healed the nerve injury. An ideal NGC would be constructed from biodegradable materials because biodegradable implants degrade naturally into compounds that our system can process without any further complications such as an unintended immune response or even poisoning. Polyhydroxyalkanoates, or PHAs for short, are a family of more than 150 natural monomers [11] found within more than 90 bacterial strains intracellularly as energy storage [12]. Among the unique properties of PHAs is their biocompatibility with numerous human cells [13] and the fact that they are biodegradable [14] which makes them a very practical extracellular scaffolding material for medical implants, for instance, they are ideal for nerve guidance applications.

In this report, we will go over the underlying mechanisms behind peripheral nerve injury and peripheral nerve regeneration. The present study will also delve into the synthetic biology process behind the production of the two Polyhyroxyalkanoates polyesters: Poly(3-Hydroxybutyrate) (P(3HB)), and a copolymer of 3-hydroxyoctanoate and 3-hydroxydecanoate: poly(3-hydroxyoctanoate-co-3-hydroxydecanoate) (P(3HO-co-3HD)). Secondly, the biocompatibility of the polyesters P(3HB) and P(3HO-co-3HD) will be investigated by seeding PHA films with NG108-15 neuronal cells and performing various biocompatibility assessments on the cell cultures, such as a live/dead analysis, a resazurin assay and a neurite extension length measurement. Then, nerve guide conduits made from various blends of P(3HB) and P(3HO-co-3HD) will be manufactured by dip coating. The conduits were then imaged through scanning electron microscopy, or SEM, to obtain detailed images of the PHA NGC's surface topography. And finally, we will

examine how nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) were encapsulated within a 50:50 blend of P(3HB) and P(3HO-co-3HD) solution that was later moulded into nerve guide conduits. The methods for sampling the NGCs for an Enzyme-linked immunosorbent assay (ELISA) will be covered, but the results in terms of the quantification of the release rate of neurotrophic factors NGF and BDNF from the conduits will not be covered.

3 – Literature Review

3.1 – Peripheral Nerve Injuries: Mechanism of Injury and Common Remedies

Our nervous system is separated into two components: the central nervous system (CNS) stretching from the brain to the spine, and the peripheral nervous system (PNS). The PNS consists of millions of nerve fibres linking the CNS to the rest of the body, the PNS is responsible for all muscle action [15]. When damage occurs to a nerve of the PNS, the communication pathways established by neural cells can become severed and can consequently result in the inability to perform simple movements and gestures, as well as the loss of sensation in the area affected. Each nerve fibre of the peripheral nervous system contains a multitude of blood vessels and axons that sprout from the body of a neuronal cell. Axon extensions are covered in many electrically insulating layers of myelin sheaths, which are synthesized by neuroglia Schwann cells (**Figure 1**). Myelination of the axon is vital to the normal function of neurones.

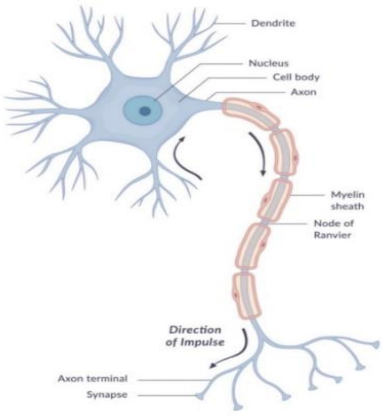


Figure 1: The anatomy of a neural cell

A distinguishable and unique property of the PNS is its ability to recover from damage through the metabolic process of Wallerian degeneration [16], [17]. Wallerian degeneration

is a self-healing process involving the total degradation of an injured nerve. This step is then followed by the promotion of axonal elongation and cell proliferation by the secretion of neurotrophic factors by Schwann cells and the formation of Bands of Büngner. Although the PNS can recover naturally from minor injuries within weeks, it is not always able to do so. The possibility of self-healing is directly correlated with the severity of damage made to the nerve [17]. This means that beyond a certain threshold, damage made to the peripheral nerve systems becomes irreversible. There is an established nerve injury grading system known as the Sunderland classification of nerve injury which is used by clinicians to determine whether surgical intervention is necessary following peripheral nerve injury [18].

This system of classification divides nerve damage into five distinct categories: Type I, II, III, IV and V injuries as shown in (**Table 1**). The first category of damage is a Type I injury which used to be referred to as neuropraxia before the Sunderland classification method. It is still referred to as such by many practicians. This type of injury often results from a compression force, that led to the deterioration of the myelin sheath surrounding a nerve's axon. Type I injuries most commonly heal without any further complications [19] whereas Type II, III and IV injuries are different classifications of what used to be called axonotmesis. Axonotmesis affects the axon and its surrounding connective tissue (**Figure 2**). To be more precise, Type II axonotmesis is the condition referring to a damaged axon within the endoneurium capsule (**Figure 2**). At this level of injury, the damaged nerve undergoes the biological process of Wallerian degeneration where the area surrounding the axon site of injury is turned into a microenvironment which is supportive of axonal elongation [17]. But before neuronal growth is promoted that is possible, the distal axonal segment and its myelin sheath begin to degenerate into small fragments. Macrophages then begin their migration to the degenerated axon site to clear up the resulting debris just a few hours following the injury [20]. And after just 24 hours following the nerve injury, Schwann cells detach from the neuronal cell's axons and begin performing two distinct roles: first, they assist the macrophages in their phagocytic activity needed to clear out the fragmented distal axon [21], and second, they begin proliferating and arranging themselves into a tube-like structure that we refer to as bands of Büngner [22]. These Schwann cell nerve conduits guide the regrowing axon in the right direction so that the nerve regeneration process is effective, while also providing an enclosed microenvironment that is promoting of neuronal cell growth [25]. The Schwann cell's phenotype undergoes drastic changes as the neuroglia cells go from a role which specialises in providing the axon with a myelin insulating layer, to one that induces axonal growth through the release of growth-promoting neurotrophic factors, that include brain-derived neurotrophic factor (BDNF), and the well-studied nerve growth factor (NGF), only to name a few [20].

Table 1: Sunderland classification of nerve injury

Classification of injury	Diagnosis
Type I	Myelin damaged
Type II	Axon and myelin damaged
Type III	Axon and endoneurium damaged
Type IV	Axon, endoneurium, and perineurium damaged
Type V	Epineurium damaged

When it comes to Type III injuries, the probability of healing occurring naturally becomes scarcer. Depending on the severity of damage made to the endoneurium layer, healing may or may not occur. For cases of PNS injuries where damage is severe enough, surgery is needed to achieve full recovery of the nerve [21]. Type IV and Type V injuries cannot heal naturally. These injuries require surgical intervention to regenerate normal nerve function, as the damage that has occurred would be beyond the body's natural capacity for repair.

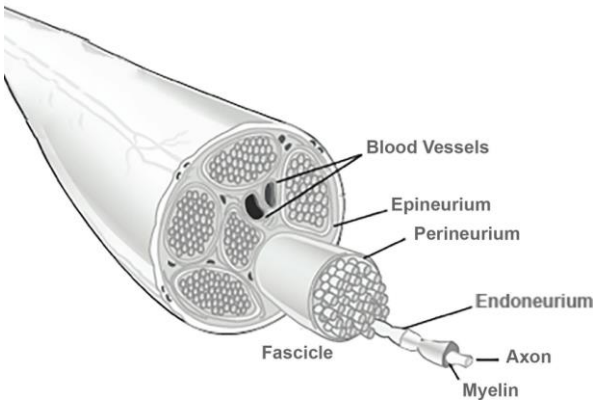


Figure 2: the anatomy of a peripheral nerve taken from [y]

As of today, the standard clinical approach for repairing nerve injuries is the autograft surgery. During an autograft procedure, healthy nerve tissue is taken from the patient's own body and is grafted on the site of injury. The surgery yields great success and is currently the gold standard procedure following a Type III, IV or V injury although it has a few distinguishable disadvantages, notably the loss of sensation at the donor site after the surgical intervention [10], the surgically invasive nature of the procedure, and tissue scarring [7]. On the other hand, nerve guide conduits (NGCs) (**Figure 3**), are a novel technology of implantable medical devices that do not lead to the unnecessary loss of sensation, and they offer a less surgically invasive alternative to Type III, IV and V nerve injuries, as these

types of implants should be designed to degrade naturally in the body, without needing additional surgery to get removed.

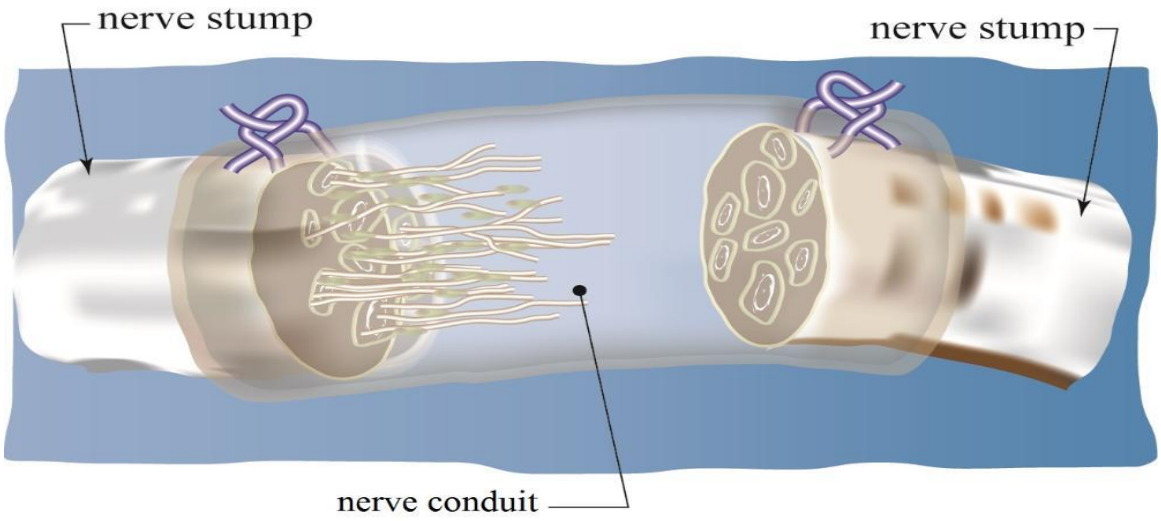


Figure 3: An illustration of a nerve conduit guiding axonal sprouts towards the other end of a severed peripheral nerve. The picture was taken from [24]

As our population continues to age, it is estimated that by 2050, 22% of the population will be over the age of fifty [25] and 1 in 5 people aged over fifty-five in the UK suffer from peripheral neuropathy, or nerve peripheral nerve damage according to the NHS [26]. Meaning that as our population continues to increase, nerve guidance could offer an alternative to autograft surgery to more than a 150 million people by the year 2050 [27] and it is worth mentioning that figure excludes the many patients that are aged under fifty-five who suffer from peripheral nerve damage [28]. Nerve guidance could have a real positive impact on the clinical experience that these patients undergo by providing them with a less invasive and more suitable alternative to nerve autografting. But to achieve this goal, to get nerve guide conduits to be considered an adequate substitute for autograft surgery, the medical device would have to be constructed strictly and entirely from biocompatible and biodegradable materials. This will ensure that the implant can resign safely within the patient's body without triggering unwanted immune responses that can compromise the patient's comfort, safety, and wellbeing.

3.2 – Polyhydroxyalkanoates

Poly(3-Hydroxybutyrate) or P(3HB) is a short-chain Polyhydroxyalkanoate (PHA) that was first discovered nearing a century ago by the French researcher Maurice Lemoigne in 1926 [29]. PHAs are a family of naturally occurring biocompatible polyesters which have even been found within our blood circulation and tissues [30], as well as in multiple

microorganism species [31]. PHAs are classified according to the length of the carbon chain at their core (**Figure X**). For instance, PHAs that have three to five carbon atoms are considered SCL-PHAs, or in other words, short chain-length polyhydroxyalkanoates [32]. Poly(3-hydroxyoctanoate-co-3-hydroxydecanoate) on the other hand is an MCL-PHA.

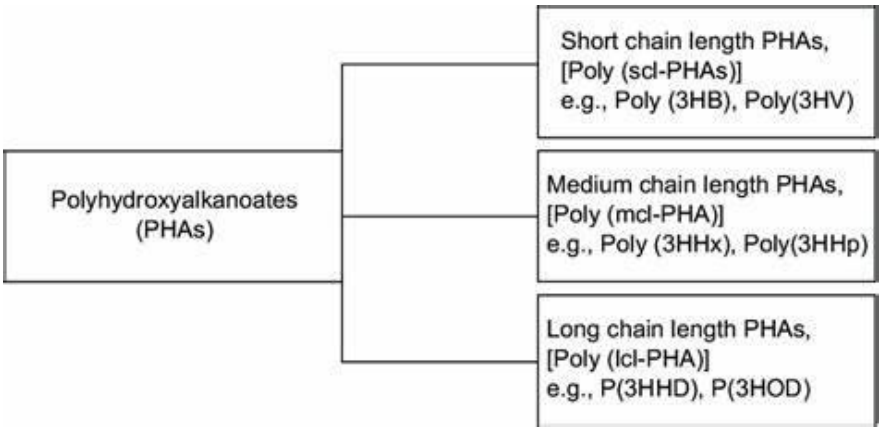


Figure 4: The standard classification of PHAs according to the length of their carbon chain [33]

PHAs are synthesized within certain microorganisms like bacteria, similarly to the way humans and animals store adipocytes or fat cells, as an energy source. Since these polymers are found within cells, they can be harvested sustainably through the means of controlled bacterial fermentation using glucose as a carbon source [34]. For instance, the PHAs studied in this report originate from respectively from bacterial strains *Bacillus subtilis (B. Subtilis)* and *Pseudomonas mendocina (P. Mendocina)*. Both of the bacterial strains are aerobic microorganism that requires oxygen to break down glucose [35], *B. Subtilis* is categorised as a Gram-Negative bacterium whereas *P. Mendocina* is Gram-Positive. Bacterial fermentation is a technique of growing microorganisms commonly used in the field of Synthetic Biology to grow microbes on a large scale by providing an environment which induces their survival, growth and proliferation. numerous institutions have used it to produce insulin, yeast and energy sources biofuels like biodiesel [36], [37]. Bacterial fermentation does not require the use of unstainable sources of energy like fossil fuels which makes PHAs sustainable materials by nature. They are environmentally friendly, sustainable, and compatible with a large range of animal and human tissue cells and safely degrade over time into non-harmful metabolites [38], [39], [40]. In other words, polyhydroxyalkanoates are sustainable to produce, biocompatible and biodegradable. They are now gaining in popularity in the biomedical field [41] as they have been used to make a wide range of implantable devices, for instance, they have been used in both hard and soft tissue engineering [42]. These bioplastics (PHAs) have been tested both *in vitro* and *in vivo* as medical implants in pre-clinical studies and yielded great results [19], [43]. Like any other technology, PHA production comes with some drawbacks. The most important one being the relatively high cost of running a bacterial fermentation. Which is the reason that many studies have experimented with using waste materials such as feedstock waste [44] or food

waste to run these fermentations, in an effort to reduce the cost associated with the industrial production of polyhydroxyalkanoates.

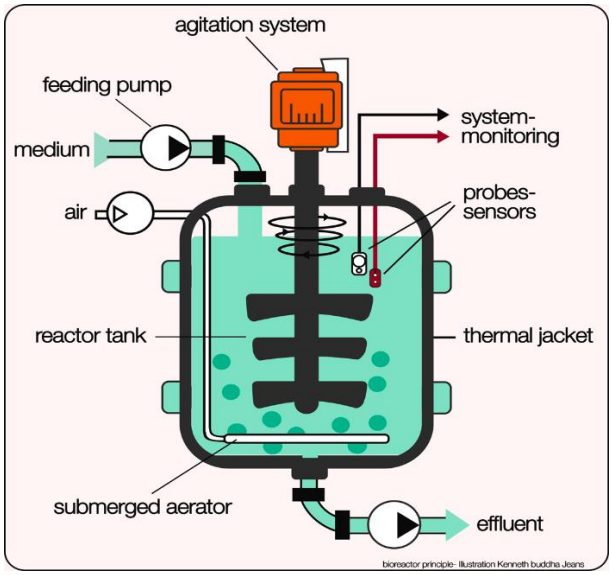


Figure 5: This diagram shows the design of the bioreactors used during the bacterial fermentation process of PHAs and other compounds of bacterial origin in detail [46]

PHAs are mixed in various blends and at different ratios to obtain a certain desired mechanical property that would mimic the structure of the organ we are aiming to substitute for. By blending PHAs we can create a new polymer with increased tensile strength, a shorter degradation period or even a higher melting point, that is not found naturally in microorganisms. In the case of nervous tissue engineering applications, for instance, nerve guidance, a blend of P(3HB) and P(3HO-co-3HD) has been shown to be effective at supporting live cultures of Schwann cells and neuronal cells as these cells of the nervous system seem to adhere and proliferate with ease on this PHA blend [46], [47]. P(3HB) is brittle and electrostatic whereas P(3HO-co-3HD) is elastomeric. When mixed at a 50:50 ratio this blend's mechanical properties have a tensile strength of 7.8 MPa and Young's modulus of 78.5, an elongation break occurring at 30% elongation, and a melting temperature of 160 degrees Celsius. These two polymers alone do not show ideal prospects for peripheral nerve therapy but when blended they become quite ideal for nerve guidance applications. NGCs made from a blend of P(3HB) and P(3HO-co-3HD) have been implanted in a 10mm median nerve defect in a rat *in vivo* and showed that they were capable of sustaining cell adhesion and proliferation and regenerating a 10mm nerve gap [19]. The nerve conduit implanted *in vivo* did not need to be removed surgically since Polyhydroxyalkanoates are biodegradable polymers that degrade through a mechanism of surface bioerosion involving hydrolysis [48],[46]. Polyhydroxyalkanoates degrade into natural metabolites which are part of our fatty acid metabolic pathway [48], [40], [1] and do not harm us in any way, they safely degrade away and get processed by our system. The longer the carbon chain of a PHA is, the longer it takes for the polymer to degrade naturally

[40]. Nonetheless, there exist techniques that ensure a faster degradation rate of PHAs if need be. PHA nerve guide conduits could be treated with ultraviolet light before being implanted in the body as treating a PHA implant with UV light has been shown to accelerate the degradation rate of these polyesters after they have been implanted in an animal [48]. This way, it is possible to ensure that the implant will not stay longer than is needed when dealing with relatively small nerve gaps. The crystallinity of the polymers is directly correlated with their rate of degradation, meaning that another way of manipulating the time it takes for implants made from PHAs to degrade in the body rate. To further emphasise the safety of PHAs, P(4HB), which is a polyester of the PHA family has already been approved by the FDA (Food and drug administration) whereas other PHAS including P(3HB) are currently awaiting their FDA approval [43].

3.3 – Neurotrophic Factors

The material a medical implant is made of isn't the only element that influences its efficiency as a medical implant. An adequate choice of scaffolding is the most essential aspect to consider when making a nerve guide conduit, the conduit's ability to promote neuronal cell adhesion, can be enhanced through encapsulation of neurotrophic growth factors, and assuring they are released at an accurate rate as advised by (de Ruiter et al., 2009). During a previous study, a PHA nerve guide conduit was manufactured and implanted in vivo in a rat, the medical implant was fully constructed from polyhydroxyalkanoates and did not encapsulate any neurotrophic growth factors. The NGC was still successful at promoting nerve regeneration by inducing cell proliferation and eventually suturing and healing the nerve injury [19]. Neurotrophic factors are molecules secreted by Schwann cells as part of the process of nerve regeneration that naturally occurs in our bodies. Nerve growth factor (NGF) and Brain-derived neurotrophic factor (BDNF) are both neurotrophic factors that are essential to the process of peripheral nerve regeneration [49]. NGF promotes axonal outgrowth [20], this is a key factor to consider because the length of a neuronal cell's axons is directly proportional to the distance being covered in the nerve by a single cell. Neurotrophic factor BDNF, on the other hand, aids in the myelination of the growing axon, following nerve regeneration which in turn contributes to the recovery of the normal function of the peripheral nervous system [20], [50]. Nerve guide conduits releasing neurotrophic growth factors at a sustainable gradient have been the subject of numerous studies [51], [52], [53], [54]. When NGF and BDNF are included in the design of nerve guide conduits, it usually translates to faster and more efficient nerve regeneration. The previously mentioned studies, and others, investigated techniques to release these growth molecules sustainably, to ensure that nerve regeneration is promoted throughout the healing process.

4 - Experimental procedures

4.1 – Bacterial fermentation

4.1.1 – P(3HB)

A culture of the *B. Subtilis* bacteria strain was inoculated with a sterile nutrient broth and grown inside an Incubator Shaker for 16 hours. Throughout the incubation period, the internal temperature of the incubator was set to 30°C and the revolution per minute (rpm) was calibrated to a value of 200. After the incubation, the *B. Subtilis* seed culture was again inoculated with a modified Kannan and Rehacek media in a 15 litre Applikon bioreactor, as described in (Basnett et al., 2021) to ensure a maximum yield of P(3HB) production. The bacterium culture was then put in the bioreactor for 48 hours, at a constant temperature of 30°C, a revolution rate of 200 rpm and an airflow rate of 1 volume of liquid per minute (vvm) to provide the bacterial biomass with oxygen. To monitor the readings of the OD450, pH, biomass concentration, ammonium sulphate concentration as well as glucose concentration were taken at timestamps: 2h, 4h, 21h, 23h, 25h, 27h, 29h, 45h and 48.5h. A graph showing the evolution of these readings was plotted using Origin.

4.1.2 – P(3HO-co-HD)

To produce P(3HO-co-3HD), a *P. Mendocina* bacterial culture was inoculated with a sterile nutrient broth and then incubated for 16 hours in an Incubator Shaker at a temperature of 30°C and revolution rate of 200rpm, similarly to the *B. Subtilis*. Following the incubation, the cells were used to inoculate a mineral salt medium designed for MCL-PHA production. The media contained sodium octanoate at a concentration of 20 mM, (NH4)2SO4 at 0.4 g/L, Na2HPO4 at 3.65 g/L, KH2PO4 at 2.8 g/L and MgSO4, 0.5 g/L [58]. The cells were then grown for 16 hours in an Incubator Shaker at 30°C and 200 rpm until they entered their mid-log phase [35]. The culture of *P. Mendocina* was finally emerged in a fifteen-litre Applikon bioreactor for 48 hours, at 30°C, 200 rpm and 1 vvm of gas oxygen [47]. Similarly, a plot showing the reading values over time was plotted using Origin.

4.2 – Polymer extraction and purification

The broth containing the bacterium culture was collected and centrifuged to separate the biomass from the rest of the media. The cells were then crushed, and later, underwent a lyophilising procedure, also referred to as a dry-freezing procedure [47]. This procedure serves the purpose of removing excess water and drying the cell biomass and consequently drying the PHA granules found within the cells. After lyophilising, the dried biomass underwent a two-stage Soxhlet purification procedure to purify the PHA polymers from

the rest of the cellular components and other impurities. The first stage was refluxing the biomass powder mixture with liquid methanol. The polymers were then extracted from the cells by refluxing the cells with chloroform at 60°C for 48 hours. Lastly, a Rotavapor R-215 rotary vacuum evaporator was used to concentrate the PHA solutions, and an ice-cold methanol liquid was used to precipitate the polymers. Following this procedure, we are left with condensed P(3HB) and P(3HO-co-HD) in their physical form, without any impurities.

4.3 – Neuronal cells NG108-15 culturing

4.3.1 – Media preparation

The media used to culture NG108-15 neuronal cells was prepared in a sterile environment and using sterilised equipment. First, 450ml of a Dulbecco's modified eagle medium was mixed with 5ml of each of the antibiotics fungizone, penicillin and glutamine. Then, 50ml of FBS (fetal bovine serum) was added to the media, resulting in a 10% FBS Dulbecco's modified eagle medium. Finally, 13mml of this media optimised for cell growth was poured into a T-25 flask, and the rest of the solution was saved for later usage.

4.3.2 – Incubation

NG108-15 neuronal cells were deposited in the T-25 flask containing 13ml of the 10% FBS solution, all within a sterile cell culture fume board to avoid the risk of contamination. The flask containing the cell culture was incubated at 37°C and monitored regularly through an AE2000 microscope. At each given time the confluency of the flask seemed to reach about 80% the old culture media was removed and the culture was further divided into three subcultures by taking three cell samples using a sterile pipette and injecting the cells into three separate T-25 flasks, also containing 13ml of the 10% FBS Dulbecco's modified eagle medium solution previously made.

4.4 – Biocompatibility assessments

4.4.1 – Making the PHA films

Five PHA blends were prepared to be used for the biocompatibility assessments with NG108-15 neuronal cells. Polymer **Blend A** was made by mixing P(3HB) and P(3Ho-co-3HD) at a 50:50 ratio, **blend B** was a 20:80 ratio of P(3HB) and P(3Ho-co-HD), **blend C** was an 80:20 ratio of P(3HB) and P(3Ho-co-HD), **blend D** was a 100% of P(3HO-co-HD)

and **blend E** was a 100% of P(3HB). Three 5% weight solutions of each of the blends were made by weighing the appropriate quantity of the PHAs and then dissolving them in 10ml of chloroform. Once the polymers were dissolved in chloroform, the solutions were fixated using tape on a magnetic stirrer plate and left to get homogenised overnight. Once the solutions had become a homogenous mixture, they were further mixed using a Vortex-Genie 2 machine and then poured into Petri dishes which were then left to dry to form large thin films.

Table 2: The polymers & polymer blends used during this experiment

Blend	P(3HB)	P(3HO-co-3HD)
A	50%	50%
B	20%	80%
C	80%	20%
D	0%	100%
E	100%	0%
F	**TCP**	

4.4.2 – Making samples

The previously PHA films were then cut into smaller circular pieces and sterilised under UV light for 30 minutes, to disinfect the samples from any potential microbial contamination. Later, the sterile films were placed in a multi-well plate with cell culture rings that serve the purpose of reducing the surface area that cells will occupy. 15,000 NG108-15 cells were seeded into each of fifteen wells containing PHA samples, and 1ml of the 10%FBS Dulbecco's modified eagle medium was poured into each well of the plate containing a 15,000 NG108-15 cell culture. The cultures were then incubated at 37°C in a 5% CO_2 incubator. These samples were then used for a live/dead assessment, a resazurin assay and an immunolabeling staining procedure.

4.5 – Live/dead cell staining

To stain the cell culture on films (**Table 2**) for confocal imaging we first began by preparing a serum-free staining solution made by mixing 30ml of a Dulbecco's modified

eagle medium solution with 18µl of Syto-9 and 8µl of Propidium Iodide. After that, the old culture media in each of the cultured wells was removed, and then it was further washed with PBS (Phosphate-Buffered Saline) to remove any remaining media. The PBS was then removed, and we injected each sample with 1ml of the previously made serum-free staining solution, the samples were then incubated for 30 minutes at 37°C in a 5% CO_2 incubator. After thirty minutes of incubation, the samples were taken out of the incubating chamber, the staining solution was removed, and each of the samples was submerged in 1ml of PBS. The samples were then taken to the confocal microscope for imaging.

4.6 – Resazurin Staining

4.6.1 – Solution Preparation

Additionally, a resazurin assessment was carried out to quantify the amount of metabolic activity of NG108-15 cells on the polymer blends shown in **Table 2** over 7 days. To perform this metabolic assay, two 10% resazurin solutions were made, solution **A** and solution **B**. Solution **A** was made by adding 3ml of resazurin stock to 27ml of a 10% FBS Dulbecco's modified eagle medium while Solution **B** was made by adding 3ml of resazurin stock to 27ml of a serum-free Dulbecco's modified eagle medium.

4.6.2 – Staining the Samples

On day 1 of the resazurin assay, the cell culture was taken out of incubation, the old media was removed from each well, and 1ml of solution **A** was poured into each well. A blank control well only containing solution **A** and no cells was made to keep a reference when performing the fluorescence reading. Following this, all the samples were wrapped in foil to protect them from harmful light and then stored in a 37°C, 5% CO_2 incubator for 4 hours. After the incubation period, three 200µl samples were taken from each well of the multiwell plate containing the polymer samples, as well as the blank control, and each 200µl sample was transferred into a 96 plate. The fluorescence of the samples was then analysed in an absorbance plate reader. After the analysis, the resazurin media was removed from the samples of the multiwell plate, each well of the plate was washed with PBS to remove any remaining chemicals and the cell media was renewed. A similar protocol was followed on day 3 and day 7 with the only exception that solution **B** was used to incubate the samples.

4.7 – Immunolabeling for neurite extension screening

To begin this assay, the culture media present in the NG108-15 samples (**Table 2**) was removed from each section of the multiwell plate containing the samples and washed with

PBS. Then, 1ml of (3.7% paraformaldehyde, 96.3% PBS) was injected into each well of the multiwell plate and left in incubation at room temperature for 20 minutes to fix the films onto the well. Afterwards, the 3.7% paraformaldehyde solution was removed, and the samples were washed with PBS. Following this step, 1ml of a (0.1% Triton X-100 0.9% PBS) was added and the samples were left to incubate at room temperature for 40 minutes. The solution was then washed with PBS. Following 1ml of a 3% BSA (Bovine Serum Albumin) in PBS was added to the samples and incubated at room temperature for an additional 30 minutes. After the incubation period, the solution was removed, and the samples were washed with PBS. The primary antibody that was used in this staining protocol was Anti-ß-III Tubulin. This stain was diluted in a 1% BSA in PBS solution and poured into each of the bioactive PHA film samples. The samples were then wrapped in foil and incubated at 4^{o}C for 48 hours. The primary antibody solution was then removed, and the samples were further washed with PBS. The secondary antibody solution was made by diluting Texas red IgG antibody in a PBS solution that is 1% BSA and contains DAPI (4′,6-diamidino-2-phenylindole). Following, the samples were covered in foil and incubated at 4^{o}C for 3 hours. Lastly, the samples were washed and PBS and then submerged in PBS. Images of the NG108-15 cells on polymer films were taken and neurite length was measured with ImageJ software. The average axon length was measured by averaging axon length recordings taken across the three samples made from each of the polymer or polymer blends (**Table 2**).

4.8 – Making nerve guide conduits

4.8.1 – Dissolving the polymers

Three conduits were made from three different blends of polymers P(3HB) and P(3HO-co-3HD) for this experiment. Blend 1 was made by dissolving 0.4g of P(3HB) and 0.4g of P(3HO-co-3HD) in 10ml of chloroform. For the PHA blend number 2, 0.16g of P(3HB) and 0.64g of P(3HO-co-3HD) were dissolved in 10ml of chloroform. In blend 3, 0.64g of P(3HB) and 0.16g of P(3HO-co-3HD) were mixed with 10ml of chloroform. These blends 1-3 were left on a magnetic stirrer overnight, and then further mixed with a Vortex-Genie 2 the following day.

Table 3: Details about the PHA blends used in this experiment are given below:

Polymer Blend	P(3HB)	P(3HO-co-3HD)
1	50%	50%
2	20%	80%
3	80%	20%

4.8.2 – Dip moulding the conduits

The homogenised solutions containing the PHA blend and neurotrophic factors were then moulded into the shape of a nerve guide conduits using a millimetre thick needle and a Qualtech programmable control vertical dip coater. To do so, the immersion speed of the dip coater was set to 3,000 µm/s, immersion time in the solution was set to 3 seconds, withdrawal speed was set to 3,000 µm/s and the coating time was 30 seconds. After 15-20 iterations, depending on each growth factor solution, a layer of polymer became visible on the dip-coating needle, which was then left to air dry for about twenty minutes before the conduits were removed and stored at a freezing temperature of -20ºC.

Table 4: Dip coating parameters

Immersion speed (µm/s)	Immersion time (s)	Withdrawal speed (µm/s)	Coating time (s)
3,000	3	3,000	30

4.8.3 – Scanning electron microscopy (SEM)

The moulded conduits were then imaged through a scanning electron microscope, details about scanning electron microscopy can be found in (Omidi et al., 2017).

4.9 – Moulding nerve guide conduits with NGF and BDNF

4.9.1 – Encapsulation of NGF and BDNF

Neurotrophic factors NGF and BDNF were used to make six different solutions as follows: **blend I** had 1ng/ml of NGF, blend **II** had 1ng/ml of BDNF, and blend **III** had 1ng/ml of a 50:50 blend of NGF and BDNF. Blend **IV** had 10ng/ml of NGF, blend **V** had 10ng/ml of BDNF, and blend **VI** had 10ng/ml of a 50:50 blend of NGF/BDNF. When the neurotrophic factors had a concentration of 1ng/ml, the solutions were made up of 0.49ml of PBS and 0.01ml of the growth factor. On the other hand, when the neurotrophic factors had a concentration of 10ng/ml, the solutions had 0.4ml of PBS and 0.1ml of the neurotrophic factor. Each one of the solutions was added to a 10ml chloroform solution containing 0.4g of P(3HB) and 0.4g of P(3Ho-co-3HD) and left to get homogenised on a magnetic stirrer plate overnight. The polymer solutions were then dip moulded into nerve guide conduits.

Table 5: The different NGC blends

Blend	NGF	BDNF	Concentration of neurotrophic factors (ng/ml)
I	100%	0%	1
II	0%	100%	1
III	50%	50%	1
IV	100%	0%	10
V	0%	100%	10
VI	50%	50%	10

4.9.2 – ELISA

To prepare samples for the enzyme-linked immunosorbent assay (ELISA), three samples of each of the PHA conduits with neurotrophic blends I-VI were cut with a scissor and then submerged in 1ml of PBS. Each time a sample was taken, the collected PBS solution was stored at -20 ^{o}C and the PBS was replaced. Samples for this experiment were taken on day 1, day 3, day 7, day 15, day 18 and day 24 of incubation at 37 ^{o}C to emulate thermal conditions found *in vivo*.

5 – Results (200 words)

5.1 – Bacterial Fermentations of *B. Subtilis* and *P.Mendocina*

Figure 3 and **Figure 4** plotted below show data measurements taken during the bacterial fermentation needed to produce P(3HB) and P(3HO-co-3HD). During the *Bacillus subtilis* fermentation (**Figure 4**), pH fluctuated from 5.7 to 5.2 and eventually stabilised at around 5.4 towards the end of the fermentation. The OD450 reading, the biomass concentration and the ammonium sulphate concentration increased as the glucose centration decreased over time, indicating bacterial growth. At the end of the 48-hour fermentation run, the total production yield of P(3HB) was 16.47%. The *Pseudomonas mendocina* fermentation (**Figure 5**) maintained a consistent pH of 6.7 throughout the 48 hours and a measured decrease in glucose and Nitrogen corresponded to an increase in OD450 reading and biomass concentration. The final P(3HO-co-3HD) production yield was 34.4%.

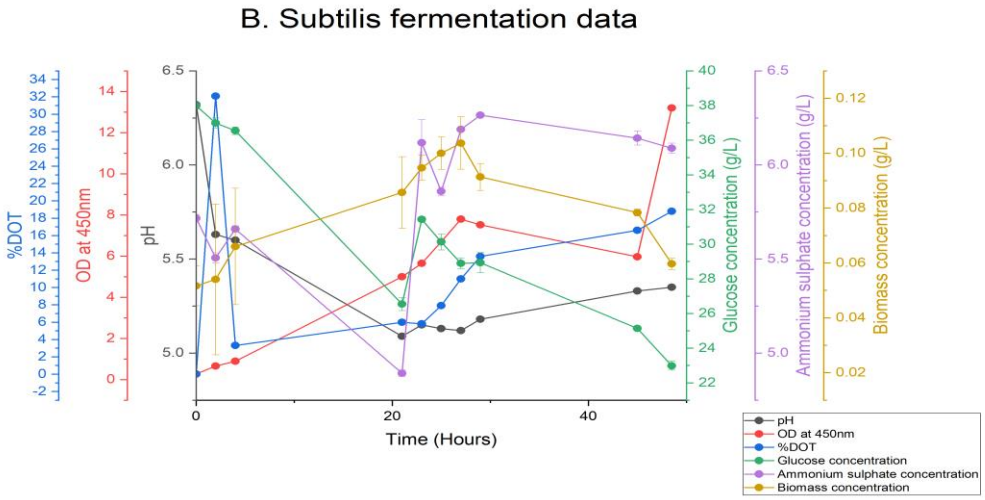


Figure 6: Data taken from the bacterial fermentation of B. Subtilis

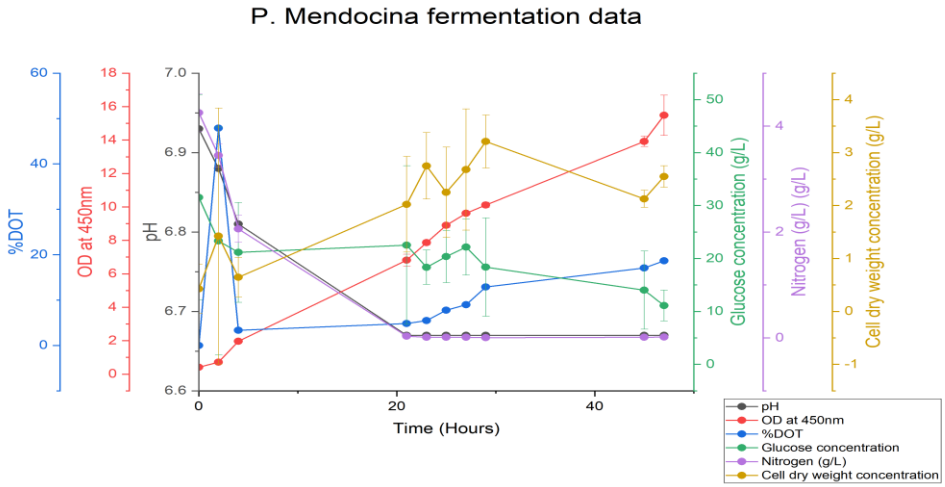


Figure 7: Data taken from the fermentation process of P. Mendocina

5.2 – Live/Dead assay

This experiment was subjected to error and out of the 6 samples stained, only 2 results from the confocal imaging were valid. The 50% P(3HB) 50% P(3HO-co-3HD) and TCP films, these samples were incubated in the same 6 well plate. The 2 other well plates were both contaminated and showed a live/dead ratio approaching 0. Results from the Resazurin Assay further prove that this cell death was caused by sample contamination and not the PHA films it was tested on.

The images of the 50:50 PHA blend are shown in the **Discussion** section, found under **Figure 11.**

5.3 – Resazurin assay

The graph below compares the measure of NG108-15 metabolic activity of the cultures seeded on PHA and TCP films. In all samples, metabolic activity peaked on day 3 of the assay. The 80% P(3HO-co-3HD) and 20% P(3HO-co-3HD) blend outperformed the rest of the tested PHA blends on day 3 and day 7 f the experiment. This polymer blend even outdid TCP on day 7, when neuronal cell activity decreased across all the samples.

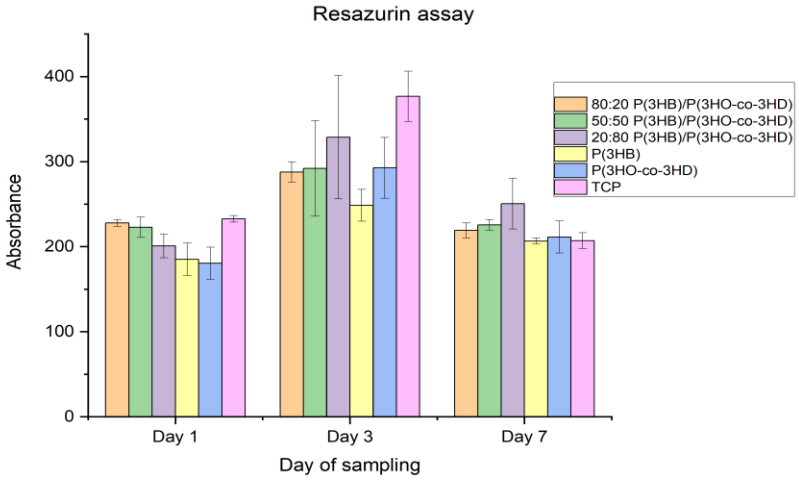


Figure 8: This plot shows an absorbance index value for each NG108 cell culture (**Table 2)**, samples were taken on days 1, 3 and 7 of this experiment

5.4 – Immunolabeling

The results from this experiment show that the *80% P(3HO-co-3HD) 20% P(3HB)* PHA blend outperformed the other **PHA** blends (**Figure 9**) in terms of average neurite extension measurement. The *20%P(3HB) 80%(P3HO-co-3HD)* blend saw the greatest amount of neuronal cell metabolic activity across samples 1 to 3. This blend was followed by the *100%P(3HO-co-3HD)* polymer, and the least quantified amount of metabolic activity was recorded on the *50%P(3HB) 50%P(3HO-co-3HD)***.**

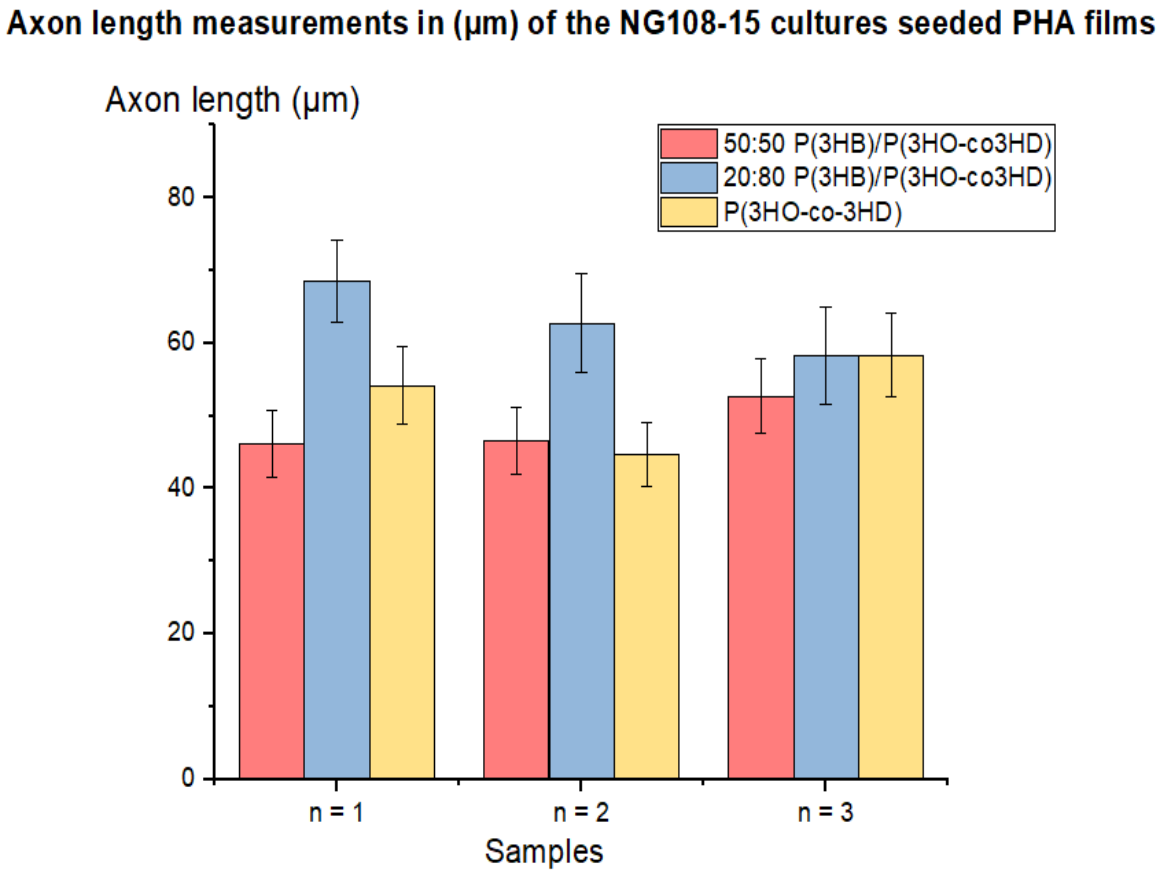


Figure 9: This figure shows the average length of the axonal extension of NG108-15 cells on PHA films

Similarly, the longest axon recorded was on the *20%P(3HB) 80%P(3HO-co-3HD)* blend **(132.79µm**), followed by the *100% P(3HO-co-3HD* where the longest axon measured *(***92.89µm** *)* and finally the longest measured axon extension on the 50%P(3HB) *50%P(3HO-co-3HD* was (**71.51µm***)* as shown in **Figure 10.**

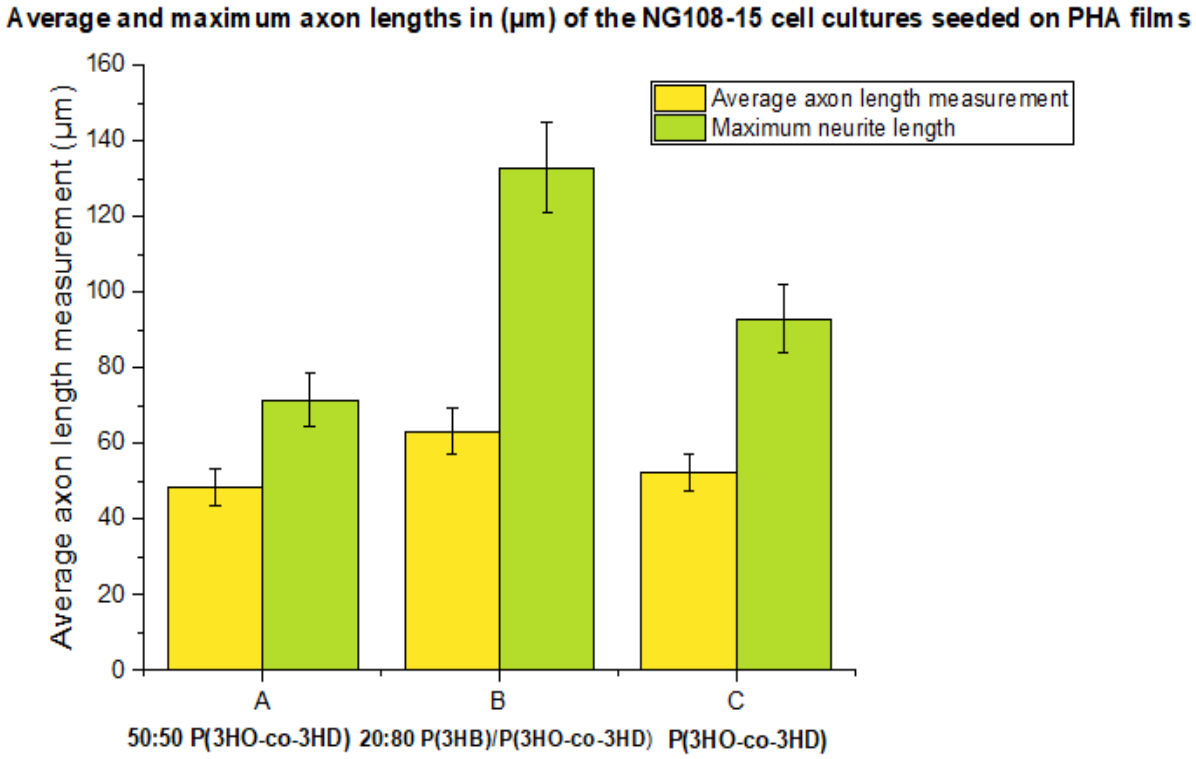


Figure 10: The graph above shows the longest axon measured in each of the PHA films **A, B and C.** As well as the average neurite extension measurements across the three polymer blends

5.5 – Scanning electron microscopy images of the nerve guide conduits

SEM images of the nerve conduits from **Table 3** are shown below in **figure 6**. The images taken at 126x magnitude showed a hollow and circular conduit with a diameter of 1mm which is ideal for nerve guidance applications. The *50%P(3HB) 50%(3HO-co-3HD)* had the thinnest conduit walls, and its surface was rather smooth, but it had a few pores on its surface. The conduit walls of the *20% P(3HB) 80% P(3HO-co-3HD)* PHA blend were thick, and the conduit's internal surface was mostly smooth. The *20% P(3HB) 80% P(3HO-co-3HD)* conduit on the other hand had many deformities. An image of a large bump inside the conduit was captured, and the conduit's surface had many clusters on it. The deformities of this conduit are most likely clusters of P(3HB).

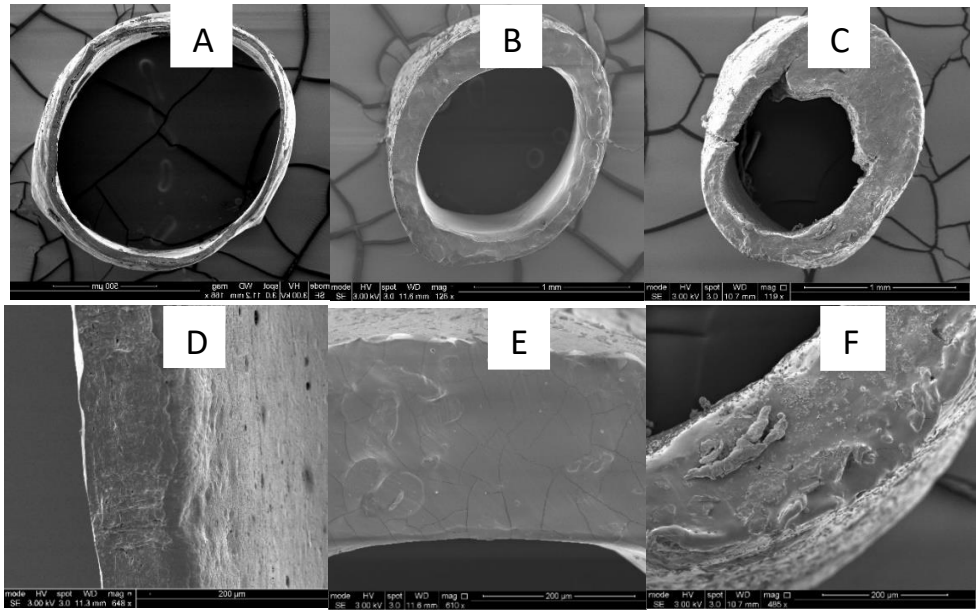


Figure 6: SEM photographs of the nerve conduits at 1mm and 200µm. **A** and **D** are images of the *50:50 P(3HB)/P(3HO-co-3HD)* conduit, **B** and **E** are the *20:80 P(3HB)/ P(3HO-co-3HD)* conduit while **C** and **F** are the *80:20 P(3HB)/P(3HO-co-3HD)* conduit

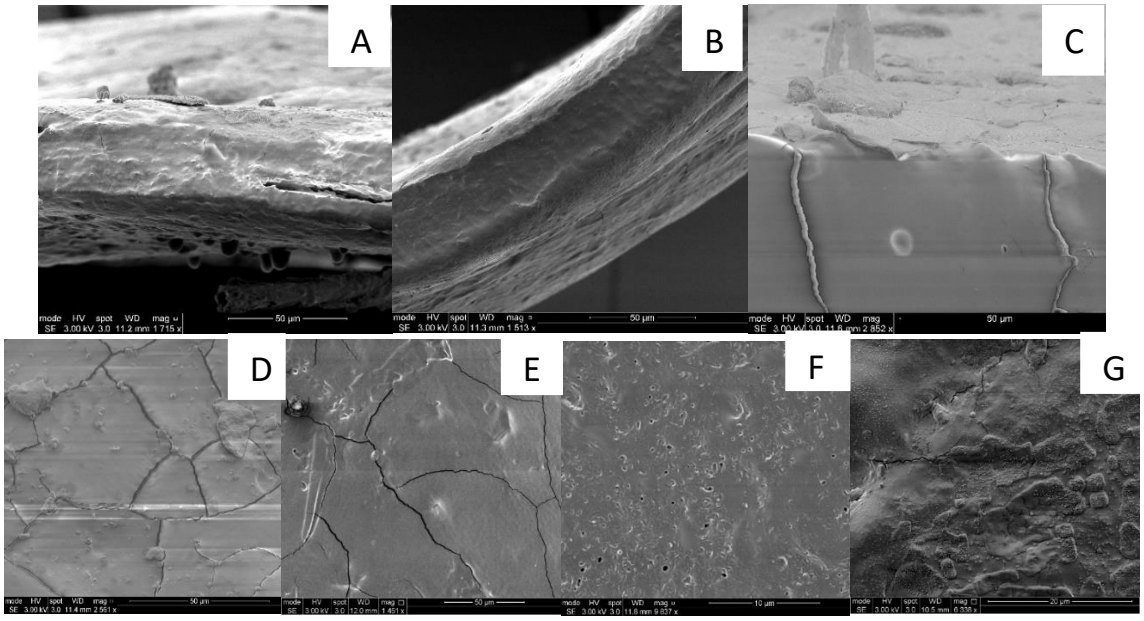


Figure 7: SEM photographs of the nerve conduits at a 50µm and 20µm scale. **A, B & F** are images of the *50:50 P(3HB)/P(3HO-co-3HD)* conduit, **(C & D)** are the *20:80 P(3HB)/ P(3HO-co-3HD)* conduit pictures while **(E & G)** are the *80:20 P(3HB)/P(3HO-co-3HD)* conduit SEM images

6 – Discussion

The work summarised in this report emphasised the biocompatibility aspect of the polyester blend of P(3HB) and P(3HO-co-3HD) by carrying out a resazurin assay and a live/dead analysis. By highlighting the synthetic biological process that produces these polyhydroxyalkanoates, and how they were extracted from the cellular walls of the microorganisms that produced them. The bacterial fermentation plots (**Figure 4** & **Figure 5)** showed that polyhydroxyalkanoates can be synthesized sustainably. Their production only requires a supply of oxygen, glucose, and nitrogen in the case *of P. Mendocina*. These compounds can be produced sustainably and ecologically, making polyhydroxyalkanoates a material of green origin. This indicates the prospect that PHAs have in modern industries that are nowadays attracted to products manufactured in a sustainable manner. Following the movement that our society is taking towards a more sustainable and environmentally friendly style of industrial production of goods. The main drawback of PHA production is still the relatively high cost involved in the bacterial fermentation process [55]. Some strategies aim to reduce this cost by using waste materials as the carbon source used to feed the bacterial strains [30], [56]. Others offer an alternative solution by maximising the yield of polymer being produced after each fermentation run, to make the process of synthesizing PHAs more cost-efficient [30]. It is crucial to begin researching novel ways to produce PHAs from microorganisms at a cheaper cost, or even to begin creating cost-efficient supply chains for PHA medical implants. Mass production strategies are known to minimise the cost of production, meaning that if PHAs were to be mass-produced and then manufactured into medical implants, then the bacterial fermentation process could be the initial costly part of a wide production plant of medical devices. The yielded economic return on investment could justify the relatively high production cost of these polymers [57].

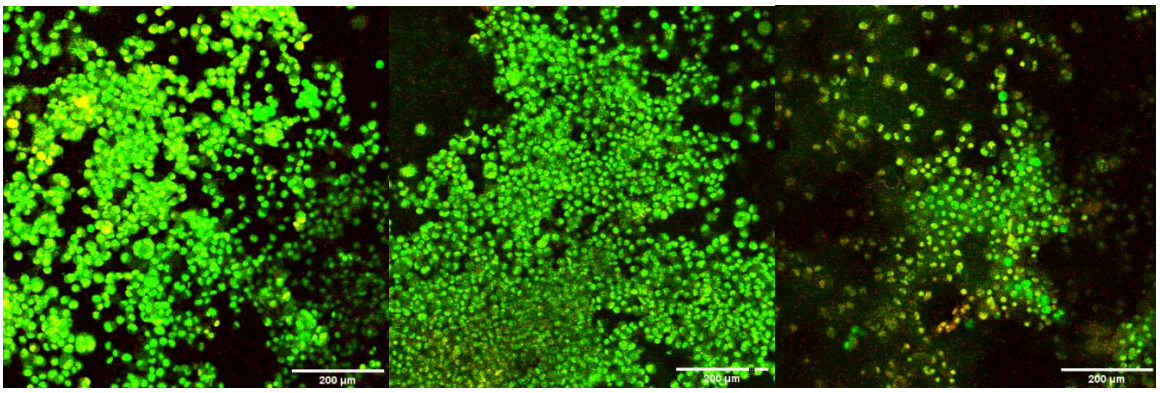


Figure 11: Live/Dead confocal microscopy images of neuronal cells NG108-15 on a 50:50 blend of P(3HB) and P(3HO-co-3HD) bacterial polyesters. Green represents healthy cells whereas red indicates a deceased cell.

Nonetheless, as the field of synthetic engineering continues to improve, and the technology behind medical devices gets more and more advanced, polyhydroxyalkanoates stand a chance to become one of the most sustainable biocompatible materials for biomedical applications on the market. A few industries are offering nerve guide conduits for purchase but none of them has used PHAs to manufacture their product, instead, most of the commercially available NGCs are made from Type I Collagen tissue [58]. It could be argued that one of the reasons we are not seeing PHA products being sold is that companies simply cannot sell polyhydroxyalkanoate based implants as most PHAs, including P(3HB), are awaiting FDA (Food and drug administration) approval [43] and therefore are not a scaffolding material option if one is looking to commercialise their medical devices. In this report, we have managed to show the great extent to which animal neuronal cells NG108-15 were able to proliferate and differentiate *in vitro* on P(3HB) and P(3HO-co-3HD) culture films. The confocal image of the 50% P(3HB) and 50% P(3HO-co-3HD) blend (**Figure 10**) image is nonetheless showcasing an impressive cell proliferation output, both in terms of survival and proliferation. Indeed, when the PHA polymers P(3HB) and P(3HO-co-3HD) are extracted and purified, they can be mixed in a chloroform solution and then extracted from the solution as a blend of the two polymers. As such, P(3HB) and P(3HO-co-3HD) blends are easily made into films by pouring the polyester mixture into a petri dish, or they can be moulded into nerve guide conduits with a dip coating machine, as shown in this work. Although dip coating is not the only way to make a nerve guide conduit, it is a simple and efficient technique, nonetheless.

This work has also shown that neurotrophic factors NGF and BDNF can be encapsulated within the PHA nerve guide conduit simply by dissolving the chemical compounds into the polyester-chloroform solution before the dip-coating step described earlier. This method of encapsulation is rapid and simple. A more advanced and complex strategy for a more effective release of neurotrophic factors over time involves immobilising the growth factors on a bioactive gel surface. A previous study has shown that proteins NGF and BDNF were successfully immobilised on an amine functionalised surface on PCL microfibres, the overall neurite extension over a period of seven days covered a total distance of 3mm. At this rate, a 1cm nerve defect could potentially get regenerated in less than 1 month. And these results were achieved by simply providing nervous tissue cells with an optimal environment for growth. These results show how effective an electrospun PHA nerve guide conduit could be at inducing peripheral nerve regeneration, especially when considering that polyhydroxyalkanoate blends are more effective at promoting neurone growth compared to PCL [59], which was the material used in the mentioned study.

Additional research experimented with immobilising growth factor NGF on a polycaprolactone membrane, the conduit was capable of sustaining cell adhesion and proliferation in a rat, consequently leading to the full recovery of a 20mm nerve gap [60]. Other related works on PHA based NGCs have shown that electrospinning the polyesters into thin microfibres yielded higher levels of cell adhesion and proliferation when tested with NG108-15 [59], [61]. Indeed, fibre diameter is directly correlated with neurite growth as it is thought that this sort of environment mimics the architecture of biological tissue, and triggers cell adhesion [59], [61]. Furthermore, some have investigated implanting neuroglia

Schwann cells in the design of their nerve guide conduits [62]. As previously discussed, Schwann cells play a major role in the process of nerve regeneration by creating an ideal microenvironment for cellular growth and axon elongation. These supportive neuroglia cells can change their expressed phenotype to promote neurite axon extension and cell growth by releasing neurotrophic factors such as NGF and BDNF. This growth-inducing role is crucial during natural peripheral nerve healing and unsurprisingly, when a co-culture of NG108 and Schwann cells was seeded on electrospun microfibres, the NG108 cells extended their axons further when compared to a culture of NG108 cells without Schwann cells [63]. While this report did not focus on using more advanced nerve guidance tools such as electrospinning, neurotrophic factor encapsulation on a gel surface or Schwann Cell therapy. It showed that polyhydroxyalkanoates are an excellent choice for extracellular scaffolding, in this context, bridging nerve gaps. We have proven that an 80%P(3Ho-co-3HD), 20%(P(3HB) PHA blend supports maximum cell elongation (**Figure 9 and Figure 10**) and growth (**Figure 8**). The SEM photographs taken of the conduits, shown in **Figures 6 and 7**, show that this polymer ratio moulded well into an NGC, compared to the other polymer blends that were dip-coated. This blend of PHAs had a smooth surface but was still rigid enough to have thick.

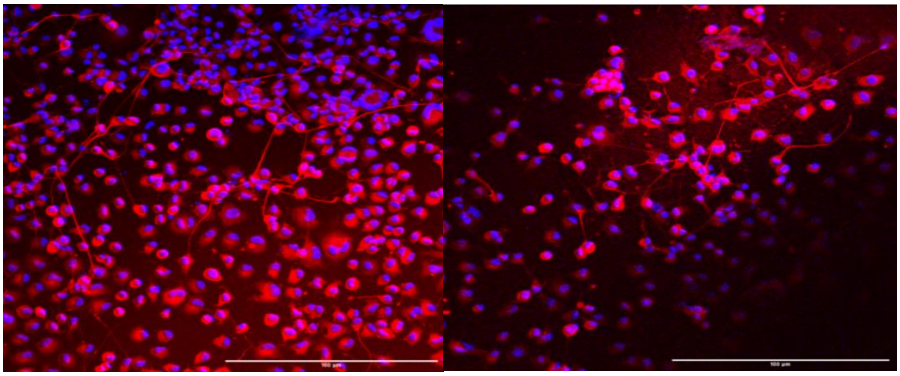


Figure 12: Confocal microscopy images showing an NG108-15 neuronal cell culture on P(3HO-co-3HD) films with cell axons highlighted in red.

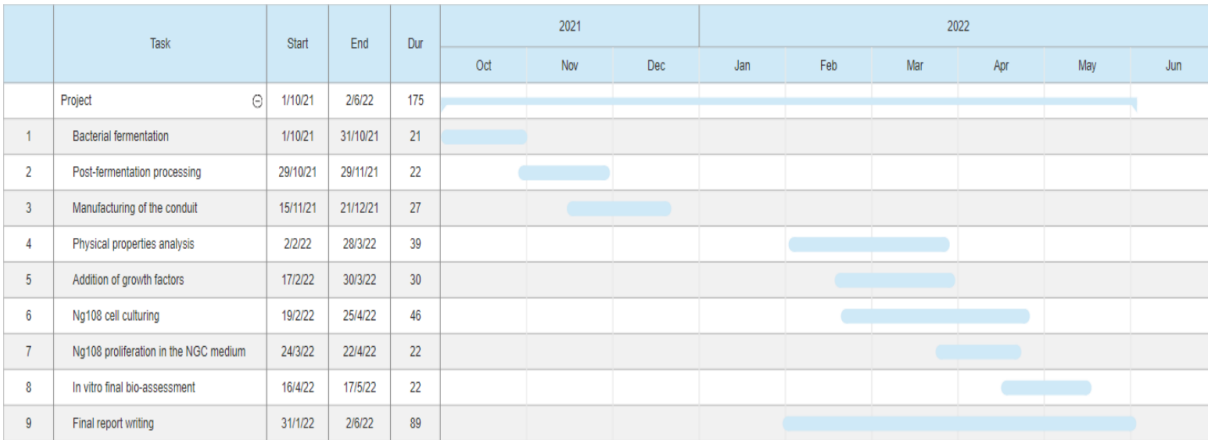
7 – Conclusions

In conclusion, we have shown the polyhydroxyalkanoate polymer blend of 20% P(3HB) and 80%P(3HO-CO-3HD), is optimal for nerve guidance applications in terms of NG108-15 neuronal cell growth and axon elongation. We have also shown that this polymer blend can easily get manufactured into a nerve guide conduit without defects. Further research on nerve guide conduits made from this optimal polymer blend is needed to quantify the mechanical property of the conduits and their rate of degradation *in vivo.* Since PHAs can be blended with one another to alter the biocompatibility, as well as the mechanical

properties of the final polymer blend, they hold the potential to become one of the greatest biocompatible and biodegradable materials for nervous tissue engineering. Since nerve guidance is highly effective at regenerating minor nerve defects during pre-clinical trials. The next logical step in this regard would be to experiment with regenerating larger, wider nerve gaps, which would be closer to a real model of PNS Type III, IV or V occurring in human beings following traumatic accidents. It was hypothesised during the discussion of the results that a nerve guide conduit made with electro 80%P(3HO-co-3HD), 20%P(3HB) fibres, additionally seeded with supportive Schwann Cells and a gradient sustainable release of neurotrophic factors NGF and BDNF would be one of the most efficient medical implants that promote peripheral nerve regeneration. Further experimentations could focus on combining all these tools and techniques to make a nerve guide conduit and test its efficiency at regenerating peripheral nerve damage. When such a NGC has been made it could be compared to the commercially available Collagen Type I scaffolds, this would be considered a major addition to the literature on nerve guidance conduit made from polyesters of bacterial origin (PHAs), and perhaps could constitute a major step towards accelerating the FDA approval and commercialisation of PHA and ultimately, creating a viable substitute to the highly invasive nerve graft surgery.

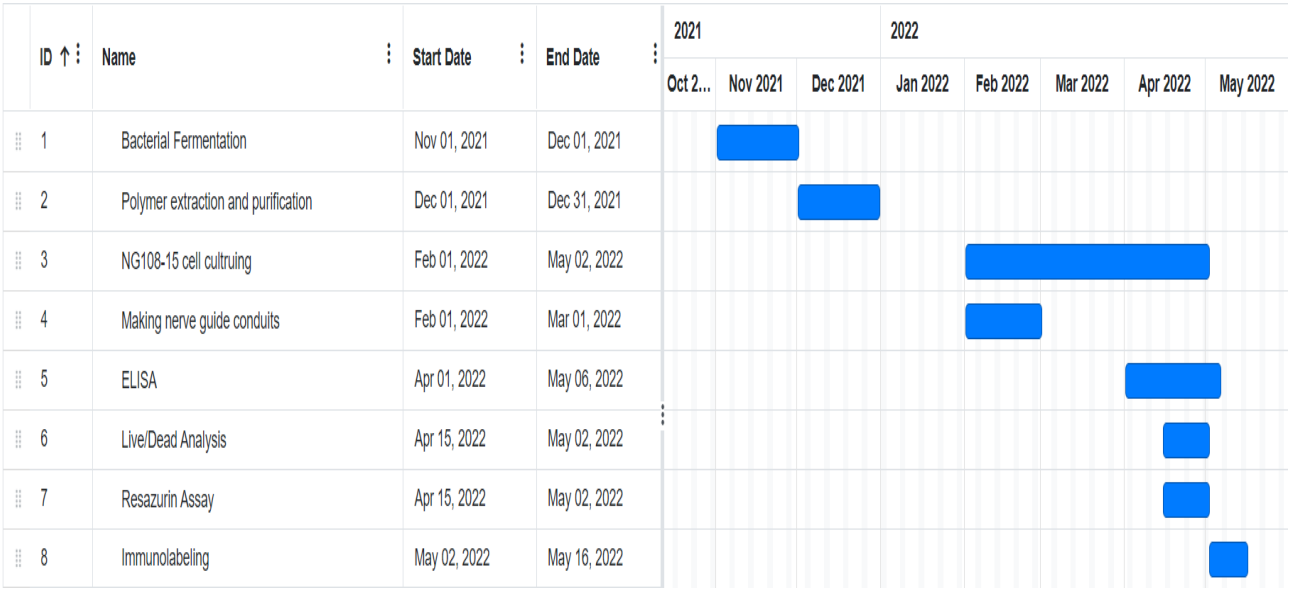
8 – Project Management:

The original plan of this project was to assess PHA nerve guide conduits with neurotrophic factors. We were planning to conduct mechanical tests that would reveal the physical properties of the dip-coated nerve guide conduits, but unfortunately, this was not possible due to the current circumstances. We also had planned to calculate the release rate of NGF and BDNF, but the ELISA kit needed to finalise the analysis did not end up getting ordered due to its high price, therefore the experiment was left half-finished. Part of the plan was to seed NG108-15 cultures on NGCs with, and without neurotrophic factors, to compare the proliferation rate of the cells in the two different mediums. Instead, we seeded the neuronal cells on a wide range of PHA blends and polymers and evaluated their biocompatibility.



	Task	Start	End	Dur
	Project	1/10/21	2/6/22	175
1	Bacterial fermentation	1/10/21	31/10/21	21
2	Post-fermentation processing	29/10/21	29/11/21	22
3	Manufacturing of the conduit	15/11/21	21/12/21	27
4	Physical properties analysis	2/2/22	28/3/22	39
5	Addition of growth factors	17/2/22	30/3/22	30
6	Ng108 cell culturing	19/2/22	25/4/22	46
7	Ng108 proliferation in the NGC medium	24/3/22	22/4/22	22
8	In vitro final bio-assessment	16/4/22	17/5/22	22
9	Final report writing	31/1/22	2/6/22	89

Figure 13: Original Gantt Chart of the project submitted in the Interim Report



ID	Name	Start Date	End Date
1	Bacterial Fermentation	Nov 01, 2021	Dec 01, 2021
2	Polymer extraction and purification	Dec 01, 2021	Dec 31, 2021
3	NG108-15 cell cultruing	Feb 01, 2022	May 02, 2022
4	Making nerve guide conduits	Feb 01, 2022	Mar 01, 2022
5	ELISA	Apr 01, 2022	May 06, 2022
6	Live/Dead Analysis	Apr 15, 2022	May 02, 2022
7	Resazurin Assay	Apr 15, 2022	May 02, 2022
8	Immunolabeling	May 02, 2022	May 16, 2022

Figure 14: Actual Gantt Chart of the project

9 – Self-Review:

Coming from the Medical Devices stream of Bioengineering, my education had mostly revolved around theoretical biology lessons and Control Systems Engineering. At the time of writing, I have come to view my Final Year Project as a year-long internship that I undertook at the Kroto Institute, working alongside great scientists and researchers. I was welcomed by the lovely people of the Roy Group, and it is through them that I have learnt about Polyhydroxyalkanoates and the drastic effect that biomaterial selection has on

cellular growth and proliferation. I have learned a lot about cell culturing, teamworking and lab ethics during my time at the Kroto Institute. Throughout the weekly meetings held each Friday, I learned to present data related to research in a way that is captivating and interesting.

Even though my project did not go as I had planned it initially in some sense, it was through dealing with this sudden change that I developed myself the most. I learnt that anything is possible if there is a will and self-confidence. Committing to writing this report greatly shaped the way I think and communicate, I have learnt the importance of presentation as well as critical analysis and scientific thinking. Finally, I would like to quote the famous Canadian academic professor Jordan Peterson as he says that writing is the same thing as thinking, and by learning to write, we learn to think.

10 – References

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