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This is an author's accepted manuscript of an article published in Journal of Materials Science: Materials in Medicine, 29, p. 179, 2018.

The final publication is available at Springer via:

https://dx.doi.org/10.1007/s10856-018-6183-9

Biosynthesis and characterization of a novel, biocompatible medium chain length polyhydroxyalkanoate by *Pseudomonas mendocina* CH50 using coconut oil as the carbon source

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Abstract

This study validated the utilization of triacylglycerides (TAGs) by *Pseudomonas mendocina* CH50, a wild type strain, resulting in the production of novel mcl-PHAs with unique physical properties. A PHA yield of 58% dcw was obtained using 20g/L of coconut oil. Chemical and structural characterisation confirmed that the mcl-PHA produced was a terpolymer comprising of three different repeating monomer units, 3-hydroxyoctanoate, 3-hydroxydecanoate and 3-hydroxydodecanoate or P(3HO-3HD-3HDD). Bearing in mind the potential of P(3HO-3HD-3HDD) in biomedical research, especially in neural tissue engineering, *in vitro* biocompatibility studies were carried out using NG108-15 (neuronal) cells. Cell viability data confirmed that P(3HO-3HD-3HDD) supported the attachment and proliferation of NG108-15 and was therefore, confirmed to be biocompatible in nature and suitable for neural regeneration.

Key Words: Polyhydroxyalkanoates, medium chain length PHAs, nerve regeneration, biocompatibility

1. Introduction

Polyhydroxyalkanoates or PHAs are a family of naturally occurring intracellular compounds synthesized by a variety of bacterial species. They are stored as carbon and energy reserves within the bacterial cells. They can be produced using a range of renewable carbon sources via bacterial fermentation. Based on the number of carbon atoms within their monomer units, PHAs can be broadly classified into two types such as short chain length PHAs (scl-PHAs) and medium chain length PHAs (mcl-PHAs). Their physical properties differ based on their type. Scl-PHAs contain 3-5 carbon atoms in their monomer units and except for Poly(4-hydroxybutyrate) or P(4HB) are brittle, stiff and have a high melting point and glass transition temperature, whereas mcl-PHAs containing 6-14 carbon atoms within their monomer units are soft, elastomeric and have low crystallinity, melting point and glass transition temperature. Optimum properties for the desired application can be achieved via blending, functionalization or by developing composites [1, 2]. Among the variety of bacterial species known to produce PHAs, *Bacillus* species and *Cupriavidus necator* are generally known to produce scl-PHAs whereas *Pseudomonas* species are known to produce mcl-PHAs. *Alcaligenes eutrophus* and *Rhodococcus* sp are able to produce PHAs containing both scl and mcl PHAs. PHAs are biodegradable and biocompatible in nature. They have gained fresh impetus in recent years for their use in biomedical applications. *In vitro* and *in vivo* studies have substantiated the ability of

these polymers to be accepted when used as implantable devices and to support the growth of various mammalian cells [3-10]. Both scl and mcl-PHAs have been used for various biomedical applications such as tissue engineering, medical device development and drug delivery studies. Cardiac patches, various drug carriers, vascular grafts, nerve conduits, heart valves, artificial blood vessels, subcutaneous implants, orthopaedic pins, stents, wound dressings, slings have been developed based on PHAs [11,12]. Moreover, P(4HB) has obtained FDA approval for clinical application as absorbable sutures in 2007 [13]. Based on their physical attributes, most widely explored scl-PHAs such as Poly(3-hydroxybutyrate), P(3HB) have been predominantly used for hard tissue regeneration such as bone substitutes or in drug delivery applications, whereas mcl-PHAs such as Poly(3-hydroxyoctanoate) or P(3HO) have been used primarily for soft tissue engineering such as cardiovascular, neural or in wound healing applications. Not only polymers but also their derivatives have been used for biomedical applications. For example, oligomers of mcl-PHAs were added as plasticisers to P(3HB) films and used in the development of tissue engineering scaffolds [14]. Methyl esters of 3HB were used as drugs against Alzheimer's disease, by preventing damage of the mitochondria [15]. In another study, methyl esters of 3HB also showed an anti-osteoporotic effect in mice and helped to maintain bone mechanical properties and structure [16]. Finally, sodium salts of 3-HB monomers have been recognised as enhancing memory agents [17].

Recently PHAs have been investigated as materials for nerve tissue engineering. Up to date, standard autologous nerve grafts have been considered as the gold standard in clinics to support peripheral nerve regeneration. However, this method is associated with several drawbacks, such as limited number of donors, scar tissue formation, loss in donor nerve function, atypical regeneration. Hence, there is a high demand for a biodegradable material suitable for neural cell support and regeneration. Currently, members of the PHA family that have been investigated in this context including P(3HB), P(3HB-co-3HV), P(3HB-co-3HHx), P(3HB-co-3HV-co-3HHx), P(3HB-co-4HB), P(3HO)/P(3HB) blends, showing suitability of PHAs for their application in nerve regeneration.

PHAs are known to have several advantages over the synthetic polymers, such as poly(caprolactone) (PCL), poly(lactic acid) (PLA) or poly(lactic acid-co-glycolic acid) (PLGA). Polyhydroxyalkanoates have been confirmed as biocompatible, biodegradable, non-toxic and non-carcinogenic [18,19]. PHAs degrade into less acidic degradation products as compared to PLA and PLGA, thereby eliminating the risk of inflammatory response. In contrast to most of the synthetic polymers, which degrade via bulk degradation, PHAs degrade via surface erosion in which the degradation occurs in a controlled manner. Controlled degradation is crucial in maintaining the integrity of implants under *in vivo* conditions. All PHAs have been intensively studied *in vitro* and *in vivo* and confirmed to be biodegradable. Also, PHAs degrade much slower in comparison to PLA or PLGA and hence can be used for long term applications. The degradation rate of polyhydroxyalkanoates can be manipulated by blending, either with small molecular weight PHAs or other fast degrading polymers [11].

PHAs have been deemed as potential substitutes for their synthetic counterparts derived from non-renewable petroleum reserves. However, one of the major deterrents in the commercial exploitation of PHAs is its production cost. The most expensive factor in the production of PHAs is the cost of the carbon source (40%-50% of the total) and the downstream process (30% of the total). To circumvent this, one of

the strategies involved in the economical production of PHAs is the use of inexpensive, renewable carbon feedstock. Numerous inexpensive carbon substrates such as tallow, soy molasses, agro-industrial waste, wastewater, whey, plant oils and molasses have been investigated for the production of mcl-PHAs using a range of bacterial strains. Among these, plant oils represent a new and attractive option. As a mixture of fatty acids, they are suitable for PHA production and are cheaper than the purified acids. They are a competitive alternative to the sugars as they possess higher carbon content per weight indicating that a higher polymer yield could be obtained using such oils as substrates. Plants oils are also comparatively cheaper compared to most common sugars [20-22].

In this paper, we report the production of a novel mcl-PHA by *Pseudomonas mendocina* CH50 using coconut oil as the sole carbon source and establish its biocompatible nature proving its suitability for biomedical applications, especially neural tissue engineering.

2. Materials and Methods

All chemicals used in this study were purchased from Sigma-Aldrich (Dorset, UK), VWR (Poole, UK), Lonza (Slough, UK) and Thermo Fisher Scientific (Dartford, UK).

2.1. Production of PHAs by *Pseudomonas mendocina* CH50

PHAs were produced by *Pseudomonas mendocina* CH50 (NCIMB 10541) using 20 g/L of coconut oil as the sole carbon source. The production was carried out in a 20L bioreactor and in batch mode in two stages [5, 23, 24]. The temporal profile of the production was obtained by monitoring optical density (OD_{450}), biomass, nitrogen, pH and dissolved oxygen tension (%DOT) throughout the course of the fermentation. Nitrogen was measured using the phenol hypochlorite method [25]. PHA was recovered from the freeze dried biomass using a two-stage Soxhlet extraction method using methanol and chloroform. The polymer was precipitated in ice-cold methanol under stirred conditions.

2.2. Characterization of produced PHAs

Structural identification of the produced PHAs was investigated using GC-MS (Varian GS/MS system with Chrompack CP-3800 and Saturn 200 MS/MS block) and ^{13}C and ^{1}H NMR spectroscopy (Bruker Avance III 600 Cryo).

Thermal properties of the PHA were determined using DSC 214 Polyma, Netzsch, Germany. The thermograms were analysed using PROTEUS 7.0 software.

The molecular weight of the polymer was studied using GPC (1260 Infinity II GPC/SEC system) (Agilent, Stockport, UK), equipped with PLgel 5µm MIXED-C. The data were analysed using “Agilent GPC/SEC” software.

2.3. Preparation and characterization of solvent cast PHA films

Solvent cast PHA films were produced using 5% w/v polymer solution in chloroform. The solution was poured into glass petri dishes and allowed to dry at room temperature [6].

The mechanical properties of the material were studied using tensile testing (5942 Testing Systems, Instron) using 3 polymer film samples of 5 mm width and length of 3.5-5.0 mm, gauge length 25 mm, applying a deformation rate of 10 mm per minute. The data was analysed using BlueHill 3 software.

The surface topography of the PHA films was studied using SEM (FEI XL30 FEG, Eindhoven, Netherlands). Static contact angle study was carried using the KSV Cam 200 optical contact meter [6]. These analyses were carried out at the Eastman Dental Institute, University College London, UK. Protein adsorption on the PHA film was quantified using the Bicinchoninic acid assay [5].

2.4. *In vitro* cell studies

NG108-15 (mouse neuroblastoma x rat glioma hybrid) cells were cultured using Dulbecco's modified Eagle's medium (DMEM) with 10% FBS at 37°C at 5% CO_2 in a humidified incubator.

2.4.1. Indirect cytotoxicity of PHA films

1cm^2 film samples were UV sterilized for 30 minutes on each side and incubated in DMEM medium for 24 hrs at 37° C at 5% CO_2 in a humidified incubator. NG108-15 cells (20,000 cells/ mL) were seeded in a 96-well plate and incubated for 24 hrs in DMEM medium. The media was replaced with the extracts obtained after the incubation of the polymeric films and the cells were cultured for 24 hours. Cell viability assay was carried out using Alamar Blue assay (Thermo Fisher Scientific). Standard tissue culture plastic (TCP) was used as the positive control.

2.4.2. Cell proliferation studies

NG108-15 cells (cell density – 25,000 cells per well) were seeded on to the UV sterilized 1cm^2 P(3HO-3HD-3HDD) solvent cast films and incubated for a total period of 7 days. Cell viability tests were carried out on day 1, day 3 and day 7 using the assay described above. TCP was used as the positive control. To study the morphology of the cells seeded on the PHA film, the cells were fixed in 2% paraformaldehyde, dehydrated, gold-coated and viewed using SEM.

2.5. Statistical analyses

All measurements were made in triplicates, and data are presented as mean values ± standard deviation. One way Anova tests were performed with GraphPad Prism 6 software and differences were considered statistically significant when p-values were lower than 0.05.

3. Results

3.1. Production of PHAs by *P. mendocina* CH50 using coconut oil as the carbon source

The temporal variation of crucial parameters including OD, pH, % DOT, biomass concentration, nitrogen estimation and polymer yield were monitored during a 15L (working volume) fermentation of *P. mendocina* CH50 (Figure 1).

Fig. 1 Temporal profile of PHA production by *Pseudomonas mendocina* CH50 using mineral salt medium containing coconut oil as the sole carbon source.

It was observed that the OD increased gradually throughout the course of the fermentation and reached a maximum value of 31.0 at 48 hrs. The biomass concentration increased along with the optical density of the bacterial culture until 45 hours, reaching a maximum of 2.7g/L and then decreased up to 2.5g/L at 48h. The pH of the production medium was set to 7.2. During the fermentation, the pH slightly decreased from 7.2 to 7.0 at 48 hours. pH was not controlled during the course of this experiment. Nitrogen was the limiting nutrient in this particular study. The concentration of nitrogen decreased from its initial value of 0.12 g/L to 0.015 g/L within 27 hours indicating that a nitrogen-limiting environment was achieved during the fermentation. DOT decreased rapidly from 100% to 0% within the first hours of the fermentation creating an oxygen-limiting environment. Bacteria continued growing until the end of the cultivation as indicated by the increasing OD values. Previous studies have shown that dual nutrient limitation such as nitrogen and oxygen limitation enhances polymer accumulation [26, 27]. The polymer accumulation started at 9hrs. Polymer yield increased from 1.5 % dcw at 9 hours to maximum of 58.0 % dcw after 48 hours. The highest polymer content was observed during the nitrogen and oxygen limitation phase, which is in agreement with literature [28, 29]. Coconut oil has been used previously as a carbon source for PHA production. Wong *et al.* observed the production of P(3HB-3HHx) copolymer using *Cupriavidus necator* [30], while Ashby and Foglia [31], followed by Solaiman *et al.* produced mcl-PHA copolymer [32], containing five different monomer units using *Pseudomonas resinovorans* and recombinant *Pseudomonas* strains respectively. *Pseudomonas mendocina* CH50 has been reported as a mcl-PHA producer, when cultivated on different carbon sources, such as: sodium octanoate or biodiesel waste [7-9]. The PHA produced by *Pseudomonas mendocina* CH50 using coconut oil as the carbon source was further characterised.

3.2. Characterization of produced of PHAs

Gas Chromatography Mass Spectrometry (GC-MS): GC-MS analyses of methanolysed products showed the presence of three peaks, which were identified using MS (NIST) library. The peak at the retention time (R_t) of 6.745 min was identified to be methyl ester of 3-hydroxyoctanoic acid, the peak at a R_t of 9.204 min was identified as the methyl ester of 3-hydroxydecanoic acid and the peak at R_t of 10.622 min was identified as that of the methyl ester of 3-hydroxydodecanoic acid (Fig. 2a). Thus, the polymer produced by *P. mendocina* CH50 using coconut oil as carbon source was identified as the terpolymer of 3-hydroxyoctanoate, 3-hydroxydecanoate and 3-hydroxydodecanoate, Poly(3-hydroxyactanoate-*co*-3-hydroxydecanoate-*co*-3-hydroxydodecanoate) or P(3HO-3HD-3HDD). The mole % of the monomers were 30.4 ± 2.1 mole% 3HO, 48.4± 0.8 mole% 3HD and 21.2±2 mole% 3HDD. Coconut oil contains mainly saturated fatty acids (93%), both medium (C_6-C_{14}) and long (over C_{14}) chain length. Monounsaturated and polyunsaturated fatty acids constitute only 7-8% of coconut oil. The main fatty acids are lauric acid (C12:0) (up to 55%) and myristic acid (C14:0) [33]. According to the literature *Pseudomonas* sp. readily use fatty acids for PHA production. PHAs are usually produced via β-fatty acid oxidation. As a result, fatty acids are shortened after each cycle usually by two, four, six, atoms of carbon, which leads to the production of different copolymers. The mcl-PHA produced in this study, P(3HO-3HD-3HDD) has three monomer units: C_8, C_{10} and C_{12}, which can be correlated to the major fatty acids present in the coconut oil. *Pseudomonas* sp are known to be versatile in nature and they are capable of utilizing a wide range of both complex as well as refined carbon feedstock for the production of mcl-PHAs. However, very few wild type

Pseudomonas sp such as *P. resinovorans, P. aeruginosa* and *P. saccharophila* have been shown to metabolize triacylglycerides for the production of PHAs [32, 34, 35]. A successful attempt has been made to genetically engineer *P. putida* to utilize triacylglycerides for mcl-PHA production[33]. This study has demonstrated, for the first time, production of a novel mcl-PHA using a wild type strain, *Pseudomonas mendocina* CH50, using coconut oil, thereby, increasing the number of strains within the *Pseudomonas* family that are capable of utilizing triacylglycerides for the production of mcl-PHAs. The monomer composition of the PHAs are determined by the enzymes that are involved in metabolizing fatty acids to 3-hydroxyacyl-CoA precursors, which are then utilized by the PHA synthase enzyme. It is known that the mcl-PHAs produced by *Pseudomonas* sp using triacylglycerides as the carbon source have hydroxyoctanoate (HO) and hydroxydecanoate (HD) as the dominant monomer units [32]. A similar observation was made in this particular study where 30.4 mol % of HO and 48.4 mol% of HD was obtained. Several studies that have reported the production of PHAs by *Pseudomonas* sp. using coconut oil as the carbon source, including this study, have been summarized in Table 1.

Table 1 Comparison of studies conducted on the production of PHAs by *Pseudomonas* sp using coconut oil as the carbon source.

Bacterial Strain	**Monomer composition (mol%)**								
	3HB	**3HV**	**3HHx**	**3HO**	**3HD**	**3HDD**	**3HTD**	**3HHD**	**3HOD**
***Pseudomonas mendocina* CH50**				**30.4**	**48.4**	**21.2**			
Pseudomonas resinovorans NRRL B-2649[31]			8	37	35	17	3		
P. oleovorans NRRL B-14683 recombinant strain [pCN51lip-1]c[32]			6	51	29	12	2		
P. putida KT2442 [pCN51lip-1][32]			5	46	34		2		
Pseudomonas aeruginosa MTCC 7925[36]	54.1	14.8						19	18.1

None of the previously reported mcl-PHAs produced by the *Pseudomonas* sp using coconut oil as the carbon source have the same monomer composition as the mcl-PHA copolymer obtained in this study. Song *et al*., reported the production of mcl-PHAs with identical monomer units such as P(3HO-3HD-3HDD) by *Pseudomonas* sp DR2 using citrate as the sole carbon source. The monomer composition of P(3HO-3HD-3HDD) obtained was 7.74 mol% HO, 41.14 mol% HD and 51.12 mol% HDD which differs from the monomer composition of the P(3HO-3HD-3HDD) obtained in this study [37]. Therefore, this

study demonstrated the production a novel mcl-PHA, P(3HO-3HD-3HDD) with unique monomer composition and material properties.

Nuclear Magnetic Resonance (NMR): Further confirmation of the molecular structure of the polymer was obtained using ^{1}H and ^{13}C NMR spectroscopy (Figure 2). Five peaks were identified in ^{1}H NMR (Fig 2c). The multiplet resonance centred at around δ=2.5 ppm and the signal at around δ =5.2 ppm originated from hydrogens bound to carbon atoms in the polymer backbone, namely C_2 and C_3, respectively. These peaks are characteristic of Poly(3-hydroxyalkanoates). The ^{1}H NMR is not capable of discriminating methylene hydrogen atoms in pendant alkyl chains. However, the hydrogen of the end methyl group (C_8 for 3-hydroxyctanoate, C_{10} for 3-hydroxydecanoate and C_{12} for 3-hydroxydodecanoate) were identified by the signal at δ =0.88 ppm. The peak at δ =1.58ppm was assigned to two hydrogens bound to C_4. An intense wide peak at around δ =1.27ppm originated from the hydrogen atoms bound to carbon C_5-C_7 of 3-hydroxyoctanoate, C_5-C_9 of 3-hydroxydecanoate and C_5-C_{12} of 3-hydroxydodecanoate. The peaks present in the ^{13}C NMR spectra (Fig 2b) were identified according to [38] and showed spectral lines that originated from the three monomers. Analysis of the ^{1}H and ^{13}C NMR spectra was in concordance with GC-MS, hence confirming that the polymer produced by *P. mendocina* CH50 with coconut oil as carbon source is a copolymer of P(3HO-3HD-3HDD).

Fig. 2 a) GC-MS spectra of P(3HO-3HD-3HDD) and b) ^{1}H NMR spectra of P(3HO-3HD-3HDD) and c) ^{13}C NMR spectra of P(3HO-3HD-3HDD)

Thermal and Mechanical Properties: DSC heating thermograms of aged P(3HO-3HD-3HDD) showed both glass transition and melting thermal events (Fig 3). Thus P(3HO-3HD-3HDD) is a semi-crystalline polymer. The glass transition temperature was in the range between -42°C to -45°C. The crystalline phase of P(3HO-3HD-3HDD) melted between 25 to 56°C. The highest melting rate (endothermic peak maximum) was 48±2°C. These low T_m and T_g values indicated presence of highly flexible chains and large free volume in the P(3HO-3HD-3HDD) polymer due to the presence of bulky pendant chain. The T_m and T_g values of P(3HO-3HD-3HDD) corresponded to the range of values obtained for mcl-PHAs in the literature. T_m values for mcl-PHAs generally range between 40 to 60^{o}C whereas T_g values range between -50 to -25 ^{o}C [5, 7, 8].

The DSC experiments included two heating scans. After complete melting in the first heating, P(3HO-3HD-3HDD) melt was cooled at 20 degrees per minute. Heating of the vitrified P(3HO-3HD-3HDD) in the second scan demonstrated the absence of melting event. Thus P(3HO-3HD-3HDD) is a slow-to-crystallise polymer. The degree of crystallinity P(3HO-3HD-3HDD), evaluated as enthalpy of fusion, changed in the course of polymer ageing at room temperature for 5 weeks. No further change in DSC thermograms were observed after 5 weeks. Similarly, mechanical properties of solvent-cast P(3HO-3HD-3HDD) films changed within this period of time. Thus, under these storage conditions, crystallisation of P(3HO-3HD-3HDD) completed in 5 weeks. Such slow crystallisation kinetics might require a search for additives to accelerate the crystallisation in order to achieve peak product performance quickly after fabrication. The following mechanical properties were obtained in tensile testing of P(3HO-3HD-3HDD) films aged for 5 weeks at room temperature: ultimate strength, 6.6±0.4 MPa; Young's modulus, 2.0±0.3 MPa; elongation at break, 540±20%. P(3HO-3HD-3HDD) is a relatively soft, highly stretchable polymer. Based on

literature, mcl-PHAs have low crystallinity which endows them with an elastomeric property, making them suitable for soft tissue engineering [5-8, 24]. They could also be used as an additive to improve the ductility of other stiff polymers.

Fig. 3. DSC thermograms of P(3HO-3HD-3HDD) aged for 1 and 5 weeks at room temperature.

Molecular weight analysis: Molecular weight (M_w) of P(3HO-3HD-3HDD) was found to be 333±1.4 KDa with a polydispersity index (PDI) of 2.37±0.1. Previous studies have shown that the M_w of the PHAs is determined by the type of bacterial strain, process parameters, carbon source used and their monomeric composition [39]. The extraction method is also known to influence the molecular weight of the PHAs [5]. Based on literature the average molecular weight of mcl-PHAs with both saturated and unsaturated pendant groups lies within the range of 60-410 KDa [40]. M_w of P(3HO-3HD-3HDD) obtained in this study was found to be within this range. A lower PDI value indicated uniform distribution of molecular mass within the polymer, highly advantageous for use of the polymer in prototype development.

3.3. Characterization of P(3HO-3HD-3HDD) solvent cast films

Surface Properties: Surface topography of the P(3HO-3HD-3HDD) films was studied using SEM. SEM image of the surface of P(3HO-3HD-3HDD) film is shown in Figure 3.

Fig. 4: Scanning electron microscopy images of the surface of the P(3HO-3HD-3HDD) solvent cast film.

SEM studies revealed that the surface of P(3HO-3HD-3HDD) films was largely smooth in nature. Few pores were visible across the surface of the films. This could be due to rapid evaporation of the solvent while drying. This is in accordance with literature where the surface of the mcl-PHAs has been known to exhibit relatively smooth appearance. This could be attributed to the low crystallization rate of the polymer, allowing the polymer chains to rearrange gradually avoiding the formation of protrusions on the surface of the film on drying [7, 23, 41].

Water contact angle: Static contact angle studies was used to measure the wettability of the P(3HO-3HD-3HDD) films. Water contact angle (θ) is a measure of the wettability of the surface of the material. It is known that surfaces with θ value higher than 70 degrees are known to be hydrophobic in nature whereas surfaces with θ value lower than 70 degrees are known to be hydrophilic in nature (Peschel *et al.*, 2007). θ value of the P(3HO-3HD-3HDD) films were found to be 88±2 confirming their hydrophobic nature. This is in accordance with literature, which states that the PHAs are inherently hydrophobic in nature due to the presence of the alkyl pendant groups in their side chain. Renard *et al.*, measured the θ value of the widely explored mcl-PHAs such as Poly(3-hydroxyoctanoate) or P(3HO) to be 104±2 [41]. Bagdadi *et al.*, measured a similar θ value (101±0.8) for P(3HO) [7]. θ value of the P(3HO-3HD-3HDD) obtained in this study was found to be significantly lower compared to the θ values reported for P(3HO). Previous studies have shown that the wettability properties of the material are hugely influenced by the surface roughness. Surface roughness of the P(3HO-3HD-3HDD) films could be assessed in future to understand the decrease in the θ value compared to other mcl-PHAs such as P(3HO). Within the context of biomedical applications, decrease in the hydrophobicity was considered to be an advantage as most mammalian cells are known to

prefer hydrophilic surfaces over the hydrophobic counterparts [42]. However, more recent studies revealed that low contact angle does not necessarily correlate with higher biocompatibility. The correlation between contact angle and biocompatibility depends on the type of biomaterial, device and surrounding environment. It has been found that surfaces with moderate wettability have enhanced biocompatibility in case of tissue engineering scaffolds and blood-contacting devices. Contrasting results have been obtained in studies on intraocular lenses. Protein adsorption was found to have higher impact on biocompatibility of these devices than surface wettability [43].

Protein adsorption: Protein adsorption is one of the crucial factors that affects the biocompatibility of the material [44]. The total amount of protein adsorbed on the P(3HO-3HD-3HDD) film samples was found to be 0.7±0.19 mg/cm^2. This is higher than that reported in the literature for PHAs. The average protein adsorption for PHAs was within the range 0.12-0.35mg/cm^2 [45-47]. In another study, Rai *et al.,* 2010 reported the amount of serum proteins adsorbed on the P(3HO) films to be 0.083 mg/cm^2 [8]. This is significantly lower compared to the total concentration of serum proteins observed on the P(3HO-3HD-3HDD) film samples. This could be attributed to the lower θ value obtained for P(3HO-3HD-3HDD) compared to P(3HO). It is established in literature that higher protein adsorption occurs on hydrophilic and on rough surfaces [48, 49]. Increased protein adsorption could also indicate higher biocompatibility of the P(3HO-3HD-3HDD) copolymer. Protein adsorption from body fluids occurs within milliseconds after implantation of the biomaterial. The amount of protein attached, depends on the orientation and conformation of the protein, the environment and several factors related to the biomaterial surface, such as surface chemical composition, wettability, charge, topography, material stiffness [50]. After implantation of a material, the surface is covered by a monolayer of protein. Therefore, cells do not have direct contact with the material. Cells respond to the protein layer by adhering to bioactive binding sites [51]. Protein adsorption is essential for cellular adhesion. Short-term cell attachment will not be observed until some proteins are adsorbed to the surface. Several studies have confirmed that adsorbed proteins play a crucial role in cell-biomaterial interactions. Biomaterials with low amount of protein adsorbed on their surface resulted in reduced cell attachment [52]. Biofilm formation on medical devices is a critical issue. Several studies have been carried out to understand the effect of protein adsorption on biofilm formation. Sinha *et al.,* carried out an extensive study to understand the effect of protein adsorption in relation to biofilm formation. They observed that the biofilm formation was dependent on the amount of protein adsorbed onto the biomaterial surface. It was notable that the bacterial receptors identified the difference in the surface properties of the biomaterial and modulated their genetic expression accordingly. They concluded that it was possible to develop surface properties that would allow the bacteria to modulate their genetic expression to prompt a non-biofilm mode even after protein adsorption [53]. Moazzam *et al*. observed that the biofilm formation was heavily influenced by the physicochemical properties of the biomaterial surface. They confirmed that biofilm formation could be prevented by modifying surface characteristics via coatings or by introducing anti-adhesion properties. Additionally, low surface energy coupled with nano textured morphology of the biomaterial surface could reduce bacterial attachment [54].

***In vitro* cell studies:**

NG108-15, an established neuronal cell line, was used to investigate the biocompatibility of P(3HO-3HD-3HDD) film with the mammalian cells for their potential use in medical applications.

Indirect cytotoxicity testing: Indirect cytotoxicity was carried out to investigate the potential release of toxic products from the P(3HO-3HD-3HDD) film samples. % Cell viability was comparable to the positive control, with a value of 99 ±10 (Figure 5a). This result confirmed the absence of toxic degradation by products potentially released form the P(3HO-3HD-3HDD) film samples.

Cell proliferation studies: Cell proliferation studies were conducted to investigate the attachment and the proliferation of NG108-15 on the produced material. NG108-15 cells were seeded on to the P(3HO-3HD-3HDD) film samples over a period of 1, 3 and 7 days (Figure 4b). For day 1, the cell growth on the P(3HO-3HD-3HDD) film samples was comparable to the positive control (TCP). The % cell viability was 106 ± 4. On day 3, the polymeric film samples showed significantly higher cell proliferation compared to the positive control. The % cell viability was 123.4 ± 10 (**P=0.009). A significant increase of NG108-15 proliferation on the P(3HO-3HD-3HDD) film samples was detected at day 7. The % cell viability (148 ± 7) was significantly higher on the P(3HO-3HD-3HDD) film samples than the positive control (***P<0.001). Moreover, the proliferation on the polymeric samples at day 7 was significantly higher than the proliferation at day 3 (**P=0.006) and at day 1 (***P<0.001). This result indicated that P(3HO-3HD-3HDD) produced form *P. mendocina* CH50 using coconut oil as the carbon source favours the attachment and the proliferation of NG108-15 cells. Few studies have been conducted on the use of PHAs as nerve substitutes. Lizarraga *et al*. observed that P(3HO)/P(3HB) blend films favoured growth and differentiation of NG108-15 cells under *in vitro* conditions [55]. *In vivo* studies have also been conducted using PHAs. P(3HB) conduits have shown to repair nerve gaps of 10 and 40 mm in rat sciatic nerves and rabbit peroneal nerves, respectively [56]. Hollow P(3HB-3HHx) conduits have also been used to bridge 10 mm defects in rat sciatic nerves [57].

Fig. 5 a) Indirect cytotoxicity studies of P(3HO-3HD-3HDD) film samples using NG108-15 b) Cell proliferation study of the NG108-15 cells on the P(3HO-3HD-3HDD) film samples on day 1, 3 and 7 (n = 3). All the film samples were measured relative to the tissue culture plastic (TCP), which were normalized to 100% (n = 3). c-f) SEM images of the NG108-15 cells on the P(3HO-3HD-3HDD) film samples on day 3 (c–d) and 7 (e–f).

SEM studies: NG108-15 cells seeded on the P(3HO-3HD-3HDD) film samples were fixed at day 3 and 7 and viewed under SEM. SEM images presented in Fig 5 (c-f) revealed that NG108-15 cells adhered and proliferated evenly across the surface. For both days, the analyses revealed spreading of the cells on the polymeric surface. On day 7, the film samples were covered by a confluent layer of cells, confirming the proliferation and growth. This was also a confirmation of the cell viability data. This is an encouraging result, which makes P(3HO-3HD-3HDD) a potentially novel material for nerve regeneration. For this study, the cells were not cultured in conditions inducing differentiation. Due to this, the formation of neurites could not be detected. Further studies could evaluate the differentiation of NG108-15 on the P(3HO-3HD-3HDD) material by culturing the cells in low serum differentiation media.

4. Conclusion

In conclusion, a novel mcl-PHA, P(3HO-3HD-3HDD), was produced for the first time using *Pseudomonas mendocina* CH50 with a relatively cheap substrate, coconut oil, as the source carbon source. This study demonstrated the utilisation of an inexpensive feedstock for the production of mcl-PHAs. High PHA yield of 58.0% dcw was obtained using *Pseudomonas mendocina* CH50, a wild type bacterial strain, a great advantage avoiding the utilisation of genetically modified strains which lead to complex regulatory issues in the context of biomedical applications. This outcome is extremely promising in developing an economical and sustainable mode of PHA production for a range of applications. Biocompatibility studies confirmed that P(3HO-3HD-3HDD) supported excellent attachment and proliferation of NG108-15 cells. Hence, this study reveals that the novel PHA, P(3HO-3HD-3HDD) can be explored for various biomedical applications including nerve tissue engineering, medical device development and controlled drug delivery applications.

Acknowledgments

The authors would like to thank the ReBioStent project – European Union's Seventh Programme for research, technological development and demonstration under grant agreement number 604251, HyMedPoly project - Horizon 2020 under the grant agreement no 643050 and Neurimp project under grant agreement Grant Agreement No. 604450 for the funding provided to PB, BL, EM and RN. The authors would like to thank Professor Jonathan Knowles, Eastman Dental Institute, University College London for provision of the SEM imaging facility and the contact angle measurement equipment. Finally, the authors would also like to thank Dr. Nicola Mordan from Eastman Dental Institute, University College London for her contribution in SEM imaging.

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