Binary Polyhydroxyalkanoate Systems for Soft Tissue Engineering

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Abstract

Progress in tissue engineering is dependent on the availability of suitable biomaterials. In an effort to overcome the brittleness of poly(3-hydroxybutyrate), P(3HB), a natural biodegradable polyester, and widen its biomedical applications, plasticising of P(3HB) with oligomeric substances of related structure has been studied. A biosynthesised medium-chain-length polyhydroxyalkanoate (mcl-PHA) copolymer, the plasticizer precursor, was obtained using vegetable waste frying oil as a sole carbon source. The mcl-PHA was transformed into an oligomeric derivative by acid hydrolysis. The plasticising effect of the oligomeric mcl-PHA on P(3HB) was studied via characterisation of thermal and mechanical properties of the blends in the course of ageing at ambient conditions. Addition of oligomeric mcl-PHA to P(3HB) resulted in softer and more flexible materials based entirely on PHAs. It was shown that the oligomeric mcl-PHA transformed highly crystalline P(3HB) into materials with a dominant amorphous phase when the content of oligomeric mcl-PHA exceeded 10wt%. In vitro biocompatibility studies of the new binary PHA materials showed high viability and proliferation of C2C12 myoblast cells. Thus, the proposed approach for P(3HB) plasticisation has the potential for the generation of more pliable biomaterials based on P(3HB) which can find application in unique soft tissue engineering applications where a balance between stiffness, tensile strength and ductility is
required.

**Key words:** Polyhydroxyalkanoates, scl-PHAs, mcl-PHAs, oligomeric plasticiser, soft tissue engineering

**Statement of Significance**

Polyhydroxyalkanoates, a broad family of natural biodegradable biocompatible polymers, have emerged as highly promising biomaterials both for bulk and biomedical applications. Here we describe an approach to tune the mechanical properties of stiff and brittle poly(3-hydroxybutyrate) and thereby to expand its potential biomedical applications. Plasticization, a common practice in the plastic industry to modify polymer mechanical properties, has been used very cautiously for biomedical applications due to plasticizer toxicity and migration. We have developed a plasticizer for poly(3-hydroxybutyrate) based on a structurally related but softer and pliable medium chain length polyhydroxyalkanoate. Additives of oligomeric derivatives of this polymer improved ductility of poly(3-hydroxybutyrate), greatly widening the future applicability of this well-established biomaterial. In parallel, the binary polyhydroxyalkanoate materials also exhibited improved cell attachment and proliferation, a highly desirable outcome.

**1. Introduction**

The field of tissue engineering hugely relies on polymer scaffolds which provide an appropriate environment for tissue regeneration. Selection of biomaterials for scaffold fabrication presents a complex bioengineering challenge. The biomaterial scaffold temporarily replaces the mechanical function of damaged tissue or organ, supports cell proliferation and tissue formation, and degrades at an appropriate rate avoiding interference with tissue regeneration. Although the biocompatibility of scaffold materials is a paramount requirement, mechanical properties and biodegradation profiles are crucial for the selection of scaffold biomaterials. The latter properties must be tuned to a specific tissue, its regenerative ability and ultimately the regenerative ability of the host. Responding to these challenges, a vast diversity of synthetic and natural polymers are being explored in tissue engineering.
Hence, novel biocompatible polymers, which can diversify the range of mechanical properties, biodegradation pathways and kinetics, are actively sought after to advance the area of scaffold development.

In this context, Polyhydroxyalkanoates (PHAs), natural biopolyster, are attracting growing interest in the field of tissue engineering and other biomedical applications due to the versatility of PHA molecular structures and thereby their properties. PHAs are biocompatible, non-toxic and biodegradable. Wound management, coronary angioplasty, nerve regeneration, bone tissue engineering, cardiac tissue engineering and drug delivery are some examples of biomedical applications where PHA-based materials have been explored [1].

PHAs are generated by a wide variety of bacterial species usually under nutrient limiting conditions with excess of carbon. There are several factors, which hinders the commercialization of PHAs. One of the main problems associated with this is the high production cost. Various strategies have been introduced, including PHA production from mixed cultures [2], genetically engineered microorganisms, as well as changes in fermentation strategies, to overcome this problem [1]. A substantial fraction of the production costs is associated with the cost of the carbon source [3]. Therefore, use of cheap carbon sources and waste materials as the carbon source can significantly reduce the total cost of PHA production [2-4].

The properties of PHAs depend on the numbers of carbon atoms in a monomer unit and accordingly PHAs are classified into two main groups: short chain length PHAs (scl-PHAs), which contain between 3 to 5 carbon atoms, and medium chain length PHAs (mcl-PHAs), which contain from 6 to 14 carbon atoms [5]. Scl-PHAs, are semi-crystalline polymers, have high melting temperature and usually are stiff and brittle. The exception is poly(4-hydroxybutyrate), P(4HB), a scl-PHA, which is highly elastomeric. In contrast to scl-PHAs, mcl-PHAs have low melting temperature and are highly elastomeric in nature [6].

P(3HB), a well-known representative of the scl-PHA group, and its copolymers with
3-hydroxyvalerate, P(3HB-co-3HV), are currently commercially available. Due to its high crystallinity and stiffness, P(3HB) has been predominantly considered for hard tissue engineering, such as bone regeneration and particulate drug delivery systems. Potentially, biomedical applications can be broader for more ductile P(3HB) copolymers such as copolymers of 3-hydroxybutyrate and 3-hydroxyhexanoate [7]. The mcl-PHA component(s) result in higher ductility of scl-mcl-PHA copolymers compared with neat P(3HB). However, it is still a challenge to produce such copolymers with the required yields and repeatable monomer content. Hence, such copolymers are not yet commercially available. Therefore, combining P(3HB) into multicomponent polymer blend systems is considered as an alternative route towards biomaterials with a wider range of properties, since P(3HB) production is a well-established process. P(3HB) has been blended with different synthetic and natural polymers such as starch [8], poly-ε-caprolactone (PCL) [9], poly-L-lactic acid (PLLA) [10,11], poly(butylene succinate) [12], polyethylene glycol (PEG) [13], other PHAs [14-16] to achieve materials properties for a specific application. Poor compatibility in P(3HB) blends with other polymers is well-documented including P(3HB) blends with other PHAs. Therefore P(3HB) blends are multiphase polymer systems and their properties depend on the processing method used, the material’s morphological structure and the final structure of the implantable construct.

Alternatively, P(3HB) ductility can be improved using plasticizers. Various plasticizers, predominantly ester-type substances were tested for P(3HB) and P(3HB-co-3HV) [17-21]. However, for biomedical applications, especially in tissue engineering, plasticizers used must be biodegradable and non-toxic substances. Although the issue of environmental impact of plasticizers is currently widely recognised, current literature is deficient in testing cytotoxicity of implantable plasticized polymer materials. Moreover, migration of low molecular weight plasticizers, a common problem for the plasticized polymers, is most likely to be aggravated for implanted materials due to contact with physiological fluids [22]. Plasticizer migration into surrounding tissues may not only lead to toxicity effects but also drastically alter material properties before the anticipated material biodegradation.
Increase in molecular weight of plasticizers reduces the speed of migration, which has led to the development of oligomeric plasticizers [23]. Use of oligomeric analogues of a polymer as a plasticizer could be an ideal approach since it would offer good affinity between the polymer and the oligomeric plasticizer. For example, oligomers of lactic acid have been successfully used to improve the flexibility of synthetic biodegradable PLLA [24] or PLLA/P(3HB) blends [25]. However, additives of oligomerised P(3HB) to P(3HB-co-4HB) acted as a reinforcing agent rather than as a plasticiser [26]. However, a combination of oligomerised P(3HB) and monoglyceride led to efficient plasticisation of P(3HB-4HB) [26]. Synthetic oligomeric atactic 3HB diols, which are amorphous materials, induced a significant decrease in glass transition and crystallinity of P(3HB) [27]. Oligomeric derivatives of mcl-PHAs has not been yet been explored as additives for the improvement of P(3HB) flexibility.

The central focus of this paper is to develop flexible P(3HB)-based materials using structurally analogous oligomeric additives in order to enable the application of P(3HB) in a wider range of applications where a certain degree of flexibility is desirable such as soft tissue engineering, development of biodegradable stents or nerve guidance conduits. One of the mechanisms of the plasticizing effect is based on the increase of polymer free volume, as a result, favouring the selection of bulky molecules as plasticizers. To this end, oligomeric mcl-PHAs with the same molecular backbone as P(3HB) but containing bulky alkyl side chains should work as ideal plasticizing additives. Also, this study addresses the issue of high production costs of PHAs. In an attempt to valorise waste frying oil, it was used as the carbon source in the microbial biosynthesis of a novel mcl-PHA. The biosynthesised mcl-PHA was partially depolymerised by acidic hydrolysis and used in blends with P(3HB). *In vitro* biocompatibility of the blends was tested using the C2C12 mouse myoblast cell line to evaluate the potential of the new biodegradable material in applications such as skeletal muscle, nerve and cardiac tissue engineering.

2. Materials and methods

2.1. Chemicals and reagents
All the chemicals were purchased from Sigma-Aldrich (Dorset, UK) and VWR (Leicestershire, UK). Deionised water (resistivity 18.2 mΩ cm⁻¹) was used for all experiments. Cell culture media and other reagents were purchased from Sigma-Aldrich, Gibco and Fisher Scientific. Bicinchoninic assay kit for protein adsorption was purchased from Thermo Fisher Scientific (Loughborough, UK).

2.2. Microbial PHA synthesis and polymer identification

PHAs were synthesised using *Bacillus subtilis* OK2, a generous gift from Professor Fujio Kawamura (Department of Life Sciences, Rikkyo University, Japan) and *Pseudomonas mendocina* CH50 supplied by the National Collection of the Industrial and Marine Bacteria (NCIMB 10542). A batch fermentation was conducted in a 5 L bioreactor (FerMac320, Electrolab) using Kannan and Rehacek media [28], containing 35 g/L of glucose as a sole carbon source and *B. subtilis* OK2 as the producing microorganism. Fermentation with *P. mendocina* CH50 was carried out in a mineral salt media (MSM) [29,30] with 20 g/L of waste frying oil as a carbon source. Fermentations with both strains were conducted at 30 °C, under continuous stirring, at 200 rpm and air supply at 1vvm. After 48 hours of fermentation, the biomass was harvested using centrifugation. The wet biomass was subjected to freeze drying. Polymers were extracted from the dry biomass using a two-stage Soxhlet extraction. In the first stage the biomass was depleted of organic substances soluble in methanol. This was followed by PHA extraction using chloroform as the solvent. PHAs were isolated by precipitation in chilled methanol. Chemical structures of the synthesised PHAs were identified by ¹³C and ¹H NMR spectroscopy and GC-MS analysis. The latter was conducted on samples of polymers subjected to methanolysis (modified from [31]). The GC-MS analysis was carried out using a Varian GC-MS system consisting of the Chrompack CP-3800 gas chromatograph and Saturn 200 MS/MS block. The chromatograph was equipped with a 30-meter long capillary column Elite-5MS (0.25 mm internal diameter and 0.25 m film thickness, Perkin Elmer, UK). A 1 µL sample in chloroform was injected with helium (1 mL/min) as the carrier gas. The injector temperature was 225 °C and the column temperature was increased from 40 °C to 240 °C at 18 °C/min and held at the final temperature for 10 minutes.
Molecular weights of the polymers were determined by Gel Permeation Chromatography (GPC) using Agilent 1260 Infinity system equipped with a refractive index detector. The PLgel 5µm MIXED-C (300 x 7.5 mm) column was calibrated using polystyrene molecular weight standards from 162 Da to 15 kDa. Chloroform was used as a mobile phase at a flow rate of 1 mL/min. 2 mg/mL polymer solution in chloroform were used for the analysis and the injection volume was 50 µL.

2.3. Acidic hydrolysis of PHAs

The PHA produced by *P. mendocina* CH50 was subjected to partial depolymerisation by acidic hydrolysis [32]. 2 g of dry PHA was suspended in 200 mL of 83 wt% acetic acid in distilled water under stirring. Hydrolysis was conducted at 100-105°C under reflux for 20 hours. After the reaction was completed, 50 mL of distilled water was added to the solution and the mixture was transferred into a separating funnel. The product was extracted using 300 mL of chloroform. The organic layer was collected, dried over sodium sulphate, filtered and partially concentrated using a rotary evaporator. To complete evaporation of the volatile components, the product was kept in an oven at 40 degrees until a constant weight was achieved.

2.4. Film sample preparation and characterisation

The PHA-based films were prepared by the solvent cast method using a polymer solution in chloroform with total polymer concentration of 5 w/v%. The polymer solution was poured into a glass petri dish (6cm diameter) and left covered at the room temperature until complete solvent evaporation occurred and a stable weight was achieved. This study aimed at the evaluation of the influence of material ageing on their properties. The samples were stored at ambient conditions and tested after storage for 2, 5 and 7 weeks. Characterisation of aged film samples included thermal analysis by differential scanning calorimetry (DSC) and tensile tests. The focus of DSC analysis was on characterisation of aged samples of known storage history. Therefore, all DSC data presented in the paper were obtained from a single heating run from -70 °C to 200 °C. DSC 214 Polyma (Netzsch, Germany), equipped with Intracooler IC70 cooling system, was
used to conduct temperature scans at a heating rate of 10 °C/min, under the flow of nitrogen at 60 mL/min. The Enthalpy of fusion for P(3HB) was normalised to the weight fraction of P(3HB) in a polymer blend.

Tensile testing was carried out using a 5942 Instron Testing System (High Wycombe, UK) equipped with a 500N load cell at room temperature. The test was conducted using films of 5 mm width and length of 3.5-5 cm. The thickness and width of the specimen was measured in several places and an average value was used for the calculation of the cross-sectional area. The gauge length of the sample holder was 23 mm. The deformation rate was 5 mm/min. Young's modulus, ultimate tensile strength and elongation at break were calculated from the stress-strain curve. The average values for 5 specimens were calculated.

The surface roughness of the films was measured using a Proscan 1000 Laser Profilometer (Tokyo, Japan). The laser used was model 131A, which had a measuring range of 400-600 μm, a resolution of 0.02 μm and a maximum output of 10 mW. Scans of 0.5 mm² were obtained from each sample. Nine random coordinates were selected from each specimen in order to measure the root mean square roughness (Rq).

Static contact angles were measured using KSV Cam 200 optical goniometer (Helsinki, Finland). About 200 μL of deionized water was dropped onto the surface of the films using a gas tight micro-syringe. As soon as the water droplet made contact with the sample, a total of 10 images were captured with a frame interval of one second. The analysis of the images was performed using the KSV Cam software.

A protein adsorption assay was performed using foetal bovine serum (FBS). 1 cm² samples were incubated in 400 μL of FBS at 37 °C for 24 hrs. After incubation, the samples were rinsed 3 times with phosphate buffer saline (PBS) and incubated in 1 mL of 2% sodium dodecyl sulphate (SDS) in PBS for 24 hrs, at room temperature under vigorous shaking. The amount of total protein adsorbed on the surface of the samples was quantified using the Bicinchoninic Acid Protein Assay Kit. The absorbance of the samples was measured spectrophotometrically at 562 nm, against a calibration curve, using
bovine serum albumin. The samples incubated in PBS were used as a negative control. The assay was carried out in triplicates.

2.5. In vitro cellular proliferation on the film samples

100 µL of C2C12 cell suspension (cell density - 20,000 cells/ml) were seeded onto the UV sterilized 13-mm disc cut from the polymer film sample. They were cultured for a total period of 7 days at 37 °C in a 5% CO₂ atmosphere. Cellular metabolic activity measurement was carried out at the end of day 1, day 3 and day 7 using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay. 100 µL of a 12 mM MTT solution in PBS were added to each well and the plates were incubated for two hours at 37°C. The MTT solution was replaced by 200 µL of DMSO and incubated for 10 minutes. 200 µL of the resulting solution were transferred to a 96 well plate and the absorbance at 540 nm was recorded. Standard tissue culture plate (TCP) was used as the positive control. The absorbance of the samples was normalised with respect to the positive control. Additionally, C2C12 myoblasts were visualised using SEM. C2C12 grown on PHA-based films were fixed using 4% solution of paraformaldehyde in PBS. After fixation, the samples were dehydrated by 10-minute treatment with aqueous ethanol of increasing concentration (20%, 50%, 70%, 90%) and finishing with absolute ethanol. Dehydrated samples were subjected to critical drying by immersion in hexamethyilsilizalane for 2-5 minutes and left in a fume hood for at least an hour. Samples were then coated with gold using a EMITECH-K550 gold spluttering device for 2mins and imaged using a JOEL 5610LV-SEM.

2.6. Statistical analysis

The quantitative data is represented as mean ± standard deviation (n = 3). Student’s t-test was used for statistical analysis. The results were considered to be statistically significant when the p-value was lower than 0.05 *p<0.05, very significant, when **p<0.01, highly significant, when ***p<0.001. Results with p-value higher than 0.05 were considered to have no significant difference.
3. Results

3.1. Microbial synthesis of polyesters

Two different bacterial strains, *B. subtilis* OK2 and *P. mendocina* CH50, were used for the synthesis of biodegradable polyesters. In unbalanced growth conditions bacteria of the genus *Bacillus* tend to accumulate scl-PHAs [28] while *Pseudomonas* produce a large variety of mcl-PHAs [33,34]. A two-stage batch cultivation was used for the microbial synthesis using both bacteria. As a result of bacterial adaptation to nutrient conditions at the first fermentation stage, the lag phase of the bacterial growth during the main fermentation was very short for both bacteria (Figure 1). However, as expected, initially the growth of *B. subtilis* OK2 with glucose as the carbon source was quicker than the growth of *P. mendocina* CH50 when grown using waste frying oil as the carbon source. The logarithmic phase of growth was observed until 20 and 28 hours of fermentation for *B. subtilis* OK2 and *P. mendocina* CH50 respectively. The total biomass continued to accumulate during the stationary phase of *B. subtilis* OK2, up to 30 hours, and did not decrease during the fermentation, up to 48 hours. However, there was a decline in biomass content after 30 hours of fermentation with *P. mendocina* CH50, as the bacteria entered death phase. A maximum biomass concentration of 4.8 g/L during the fermentation of *B. subtilis* OK2 was slightly higher than the amount of biomass produced by *P. mendocina* CH50 (4.3 g/L). *B. subtilis* OK2 accumulated PHA quicker as compared to *P. mendocina* CH50. However, in the stationary phase, *P. mendocina* CH50 generated PHA more efficiently than *B. subtilis* OK2. As a result, after 48 hours of incubation *P. mendocina* CH50 accumulated about 40% dry cell weight (% dcw) of PHA in comparison to 30.8 % dcw in *B. subtilis* OK2.
Characterisation of the molecular structure of the polymers were carried out using NMR and GC-MS (Supplementary information, Figures S1 – S5). The polymer extracted from *B. subtilis* OK2 was identified as the homopolymer of poly(3-hydroxybutyrate) and the polymer produced by *P. mendocina* CH50 was found to be a copolymer of four 3-hydroxyalkanoates with even numbers of carbon atoms namely 3-hydroxyhexanoate (3HHx), 3-hydroxyoctanoate (3HO), 3-hydroxydecanoate (3HD) and 3-hydroxydodecanoate (3HDD). Such copolymer compositions have been previously reported in mcl-PHA microbial synthesis using various bacterial strains and plant oils [35-38] or fatty acids [31,33] as the carbon source. The dominant monomer units in these copolymers were 3-hydroxyoctanoate and 3-hydroxydecanoate. Table 1 shows the molecular composition of the
mcl-PHA synthesised by *P. mendocina* CH50 using waste frying oil. The monomer content was derived from the GC-MS results using the calibration curve of the retention time of various methyl esters of 3-hydroxyalkanoates against the corresponding carbon atom number (Supplementary information, Figure S6). As can be seen from the Table 1, the shortest (C₆) and the longest (C₁₂) monomer units consisted of only slightly more than 10 mol% with the rest of polymer consisting of almost equal fractions of 3-hydroxyoctanoate and 3-hydroxydecanoate.

Thus *B. subtilis* OK2 was confirmed as another P(3HB)-producing strain within the genus *Bacillus*. Synthesis of P(3HHx-3HO-3HD-3HDD) copolymer by *P. mendocina* CH50 was in line with the well-documented ability of *Pseudomonas* species to accumulate mcl-PHAs [39], especially when structurally related substances such as fatty acids or triglycerides are used as the carbon source. Although waste frying oil has been explored for the synthesis of PHAs [40-43], our study reports for the first time, the production of mcl-PHAs by *P. mendocina* CH50 with the utilisation of this cheap carbon source.

**Table 1.** Monomer composition the mcl-PHA synthesised by *P. mendocina* CH50 with waste frying oil as a carbon source

<table>
<thead>
<tr>
<th>Monomer type</th>
<th>Molar content, mol%</th>
</tr>
</thead>
<tbody>
<tr>
<td>3HHx</td>
<td>7.6</td>
</tr>
<tr>
<td>3HO</td>
<td>45.4</td>
</tr>
<tr>
<td>3HD</td>
<td>41.8</td>
</tr>
<tr>
<td>3HDD</td>
<td>5.2</td>
</tr>
</tbody>
</table>

3.2. **Plasticising P(3HB) with oligomeric mcl-PHA**

Oligomeric derivatives of PHAs have attracted significant interest since they can be used as reactive components in the synthesis of biodegradable copolymers. Alkaline saponification, acidic hydrolysis, transesterification, thermal degradation, ester reduction have been used for the production of lower molecular weight PHAs with various terminal functional groups [44]. Hydrolysis of PHAs in acetic acid solutions provides mild conditions leading to a slow well-controlled depolymerisation of aliphatic
polyester chains. The acid hydrolysed P(3HHx-3HO-3HD-3HDD) was found to be a waxy material. The selected hydrolysis conditions resulted in over 20-fold decrease of molecular weight (Table 2), resulting in an oligomer of molecular weight (Mₘ) around 10 kDa which was denoted as an oligo-HA. Depolymerisation was accompanied with narrowing molecular weight distribution decreasing the polydispersity index from 3.7 to 2.2. Interestingly, no melting event was observed for neat P(3HHx-3HO-3HD-3HDD) aged for 7 weeks while the oligo-HA melted at temperatures between 28°C and 38°C (Table 3 and Supplementary information, Figures S7). However, the enthalpy of fusion was only 0.84 J g⁻¹ compared to 82.5 J g⁻¹ for P(3HB), suggesting a low degree of crystallinity for the oligo-HA.

<table>
<thead>
<tr>
<th>Table 2. Molecular weight analysis of the original P(3HHx-3HO-3HD-3HDD) and the corresponding oligo-HA using GPC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Polymer</strong></td>
</tr>
<tr>
<td>P(3HHx-3HO-3HD-3HDD)</td>
</tr>
<tr>
<td>oligo-HA*</td>
</tr>
<tr>
<td>* hydrolysed P(3HHx-3HO-3HD-3HDD)</td>
</tr>
</tbody>
</table>

In order to evaluate the potential of the oligomeric mcl-PHA derivative as a plasticiser for P(3HB), the influence of oligo-HA addition on the thermal and mechanical properties of P(3HB) was studied using films prepared by solvent casting. P(3HB) embrittlement during storage is a well-known and complex phenomenon which is mainly attributed to a secondary crystallisation and a physical ageing of the amorphous phase [45]. Therefore, the evolution of thermal and mechanical properties of neat P(3HB) and its blends with oligo-HA was monitored for a period of 7 weeks for samples stored at ambient temperature. These properties are summarised in Table 3. The enthalpy of fusion of P(3HB) from *B. subtilis* OK2 was found to increase during storage for 7 weeks, confirming the previously reported secondary crystallisation of P(3HB). The total enthalpy of fusion (ΔHₜₚₚ) was lower for all blends compared to that of neat P(3HB). Also, addition of the oligo-HA resulted in a small decrease of melting temperature (Tₘ), between 2 to 5 °C. These observations suggested the inability of oligo-HA to co-crystallise with P(3HB). Thus the melting was attributed to crystals of pure P(3HB) and the enthalpy of fusion for the blends was normalised to the P(3HB) content. Such relatively small changes in
melting temperature were found to be characteristic for P(3HB) and P(3HB-3HV) plasticisation with low molecular weight plasticisers [17, 19-21]. No changes in the enthalpy of fusion and melting temperature were observed after 7 weeks of ageing (data not shown) indicating completion of P(3HB) crystallisation within 7 weeks. As can be seen from the values of normalised enthalpy (ΔH\text{norm}) of fusion, P(3HB) in the binary blends crystallised to a lesser degree compared with neat P(3HB). A similar degree of crystallinity of P(3HB) (X_c), around 48%, was achieved in blends containing 5 and 10 wt% of oligo-HA, for samples aged for 7 weeks resulting in a 20% decrease compared to neat P(3HB). The degree of crystallinity of P(3HB) dropped even further to 37% in the blend with 20 wt% of oligo-HA. However, in contrast to the neat polymer, crystallisation of P(3HB) in blends was faster and full crystallisation was achieved within two weeks of sample storage. DSC thermograms (Supplementary information, Figures S7) of blends did not show a thermal event in the range characteristic for oligo-HA melting even in the blends containing 20 wt% of oligo-HA. Thus the thermal properties indicated that addition of oligo-HA modified both the crystalline and amorphous phases in the blends.

Table 3. Thermal properties of neat P(3HB) and its blends with oligo-HA obtained using DSC.

<table>
<thead>
<tr>
<th>Week</th>
<th>Sample</th>
<th>T_m, °C</th>
<th>ΔH\text{app}, Jg^{-1}</th>
<th>ΔH\text{norm}, Jg^{-1}</th>
<th>X_c, %*</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>P(3HB) neat</td>
<td>174.8</td>
<td>82.5</td>
<td>82.5</td>
<td>56.5</td>
</tr>
<tr>
<td></td>
<td>95/5 blend</td>
<td>174.2</td>
<td>66.9</td>
<td>70.4</td>
<td>48.2</td>
</tr>
<tr>
<td></td>
<td>90/10 blend</td>
<td>167.0</td>
<td>63.9</td>
<td>71.0</td>
<td>48.6</td>
</tr>
<tr>
<td></td>
<td>80/20 blend</td>
<td>171.4</td>
<td>43.2</td>
<td>54.0</td>
<td>37.0</td>
</tr>
<tr>
<td>5</td>
<td>P(3HB) neat</td>
<td>175.2</td>
<td>84.4</td>
<td>84.4</td>
<td>57.8</td>
</tr>
<tr>
<td></td>
<td>95/5 blend</td>
<td>170.4</td>
<td>62.6</td>
<td>65.9</td>
<td>45.1</td>
</tr>
<tr>
<td></td>
<td>90/10 blend</td>
<td>173.9</td>
<td>64.1</td>
<td>71.2</td>
<td>48.8</td>
</tr>
<tr>
<td></td>
<td>80/20 blend</td>
<td>167.2</td>
<td>43.1</td>
<td>53.9</td>
<td>36.9</td>
</tr>
<tr>
<td>7</td>
<td>P(3HB) neat</td>
<td>176.6</td>
<td>85.9</td>
<td>85.9</td>
<td>58.8</td>
</tr>
<tr>
<td></td>
<td>Oligo-HA neat</td>
<td>28.6</td>
<td>0.84</td>
<td>0.84**</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>95/5 blend</td>
<td>171.7</td>
<td>62.9</td>
<td>66.2</td>
<td>45.3</td>
</tr>
<tr>
<td></td>
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<td>43.0</td>
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<tr>
<td></td>
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<td>173.0</td>
<td>43.3</td>
<td>54.1</td>
<td>37.1</td>
</tr>
</tbody>
</table>

* crystallinity degree of P(3HB) was calculated using the formula X_c = \frac{ΔH\text{norm}}{ΔH_0} \times 100 and ΔH_0=146 J/g [46].
Ageing of the materials caused significant changes in the mechanical properties for both neat P(3HB) and its blends with oligo-HA (Figure 2). The strength and stiffness of the neat P(3HB) increased during the 7-week storage, reflecting an increase in the crystallinity degree during this period. This was accompanied with the decrease of plasticity of the material, and aged P(3HB) exhibited an elongation at break of around 5%, lower than the value of 17% for the sample aged for 2 weeks. As it was mentioned earlier, this embrittlement behaviour is characteristic of P(3HB). The plasticity of the blends also changed drastically with an increase in storage time. In fact, elongation at break of aged 95/5 blend was similar to that of neat P(3HB). However, when the content of oligo-HA exceeded 10 wt%, an improvement in plasticity was observed and elongation at break for 80/20 blend was almost 20%. Both the strength and stiffness significantly decreased when the oligo-HA was added to P(3HB). Just a 5 wt% addition of oligo-HA decreased the ultimate tensile strength and Young’s modulus by 35% and 28%, respectively. The decrease in strength and stiffness further progressed with the increase in oligo-HA content of up to 20 wt%. Both the Young’s modulus and tensile strength decreased by approximately 70% for the 80/20 blend compared with that of neat P(3HB); the Young’s modulus decreased from 1.44 to 0.48 GPa while the ultimate tensile strength decreased from 20.1 to 6.4 MPa. Interestingly both the tensile strength and stiffness of aged samples was higher for the 90/10 blend compared to the 95/5 blend despite the lower content of P(3HB). At the same time, 90/10 blend was more pliable with elongation at break 6.2% compared with 3.8% for 95/5.
Figure 2. The evolution of ultimate tensile strength (a), Young’s modulus (b), elongation at break (c) during ageing of neat P(3HB) and its blends with oligo-HA.
It is worth noting that unlike the crystallinity change, the mechanical properties of the blends notably changed during the whole period of sample ageing. Thus even when crystallinity did not undergo further changes the mechanical properties continued to change. This implies that in the blends, maturation of the amorphous phase, which constituted more than 50% of the blend, continued throughout the storage period. Similar to crystallinity, no further changes in the mechanical properties of the blends were observed after storage for more than 7 weeks (data not shown).

3.3. Surface properties of 2-D scaffolds based on the binary PHA systems

SEM and laser profilometry were used to examine the topology of the polymer films. As can be seen from the SEM images presented as inserts in Figure 3, all the materials had similar surface topography with irregular protuberances and deep wells, almost circular in shape. The latter could be parts of interconnected pores. Generally, the surfaces of all materials appeared to be rough. Roughness was further characterised quantitatively as the root mean square roughness (Rq) using laser profilometry. The roughness of the neat P(3HB) film was 0.64±0.04 μm. With the increase in content of oligo-HA in the blends, the surface roughness increased from 0.83±0.02 μm for the 95/5 blend to 1.22±0.02 μm for the 80/20 composition. Thus, roughness of the films prepared using the blends was significantly higher than that of neat P(3HB).
Figure 3. Surface roughness of neat P(3HB) and its blends with oligo-HA. SEM images of the surfaces of the corresponding materials are presented as inserts.

Water contact angle and protein absorption represent physiologically relevant material surface properties. P(3HB) is a hydrophobic biopolymer and the P(3HB) films exhibited a water contact angle of 87.5±7.4° (Figure 4). According to Figure 4, there was a constant increase in the values of the water contact angle for the blends with an increase in the content of the oligo-HA, reaching a value of 118±7° for the 80/20 blends. All blend films had a statistically significant higher contact angle value in comparison to that of neat P(3HB). Obviously, the long hydrophobic aliphatic chains of oligo-HA contributed to the increase of hydrophobicity of the binary PHA blends.
Figure 4: Static water contact angle measurements for neat P(3HB) film and its blends with oligo-HA (n=3).

The affinity of the materials towards proteins was evaluated by the bicinchoninic acid assay with FBS as a model protein (Figure 5). It was found that total protein adsorption was 90±30, 105.6±15, 145±35 and 300±35 µg/cm² for the neat P(3HB) film and 95/5, 90/10 and 80/20 blends, respectively. There was no significant difference between P(3HB) and the 95/5 blend. However, there was a significant difference between the 90/10 and 80/20 blend in comparison to the neat P(3HB) film (**p=0.006 and ***p=0.0001 respectively). The amount of protein adsorbed on the film samples increased with the addition of the oligo-HA and was almost three times higher for the 80/20 blend compared with that of neat P(3HB). The higher affinity of the blend film samples to proteins is in agreement with the fact that albumin, the main component of foetal bovine serum (FBS), tends to attach better on hydrophobic and rough surfaces [47, 48].
3.4. In vitro biocompatibility studies

Preliminary in vitro cell culture studies were carried out with a mouse myoblast cell line C2C12. The central aim of this work was to expand the range of mechanical properties of bioresorbable materials based on P(3HB) and thereby to diversify their biomedical applications. Therefore, C2C12 was chosen to investigate the possibility of using softer P(3HB)-based materials, with improved plasticity, as materials for scaffolds in soft tissue engineering applications. The MTT assay was used to evaluate cell adhesion and proliferation. Attachment and proliferation of C2C12 cells on the film samples were studied over a period of 1, 3 and 7 days. The standard tissue culture plastic was used as the positive control for these experiments. The results of biocompatibility studies are presented in Figure 6.
Figure 6: C2C12 cell proliferation on the neat P(3HB) film and its blends with oligo-HA on day 1, 3 and 7 (n=3). Viability of the C2C12 cells was evaluated with respect to plastic tissue culture plate (TCP).

Cell proliferation on the P(3HB) film samples was 118.9±0.7% on day 1, 153±1.2% on day 3 and 143.1±0.7% on day 7 compared to the TCP. The differences were not statistically significant. The growth of C2C12 cells on the P(3HB) film was higher at every time point in comparison with that of TCP. The growth of the C2C12 cells on the surface of 95/5 blend was similar to TCP on day 1 and 3 but increased to 152±4% on day 7. The differences were however not statistically significant. C2C12 cell viability on films of 90/10 blend was also higher at every time point in relation to TCP: 112.9±0.4%, 118.2±0.8% and 139±1.6% on day 1, 3 and 7 respectively. The differences were however not statistically significant. The highest viability of the C2C12 cells was observed on the surface of the 80/20 blend which supported 153.9±0.2%, 159±1.4%, 177±1.5% of viable cells compared with that on TCP on day 1, 3 and 7 respectively. The differences were statistically significant in comparison to TCP on day 1 (**p=0.0016), day 3(***p=0.0001) and day 7(*p=0.0931).

Cell adhesion and proliferation was further characterised using SEM imaging. SEM images presented in Figure 7 revealed that C2C12 cells adhered and proliferated evenly across the surface. In the blends, as opposed to the neat P(3HB) films the cells seem to be more elongated and
tending towards confluency. The observed cell density increased in the order, neat P(3HB) < 95/5 blend < 90/10 blend < 80/20 blend, in agreement with the results of the MTT assay. This trend of the materials’ ability to support cell proliferation also matched the trend of increasing surface roughness, hydrophobicity and protein absorption. In summary, all the PHA-based materials supported C2C12 cell attachment and proliferation, demonstrating good biocompatibility. Addition of the oligo-HA improved cell proliferation with the highest cell viability observed for the 80/20 blend.

Figure 7: SEM images of the surfaces of neat P(3HB) and its blends with oligo-HA after culturing C2C12 cells for 1 (a), 3 (b) and 7 (c) days.

4. Discussion

This study focused on the much-needed expansion and diversification of biodegradable materials for a range of applications in regenerative medicine and other biomedical applications, including the production of bioresorbable scaffolds. *B. subtilis* OK2, a Gram-positive bacterium, was successfully used for the synthesis of P(3HB). *B. subtilis* OK2 is considered as a GRAS organism and in contrast to Gram-negative bacteria does not contain lipopolysaccharides, known endotoxins, as structural components. Therefore P(3HB) produced using *B. subtilis* OK2 minimizes the risk of material-mediated immunogenic reactions in humans.

However, embrittlement of P(3HB) is a known phenomenon which limits applications of P(3HB) including biomedical applications. To address this issue, plasticizing of P(3HB) was explored for the
development of softer and more flexible materials based entirely on PHAs. In order to maintain the biocompatibility of the materials our central aim was to test a biodegradable plasticizer derived from mcl-PHAs. For the first time, microbial synthesis of mcl-PHA was achieved using *P. mendocina* CH50 bacteria with waste frying oil as the sole carbon source. Utilization of waste materials would significantly contribute to the reduction of the overall costs for microbial PHA production. The synthesized mcl-PHA was a copolymer of 3-hydroxyhexanoate, 3-hydroxyoctanoate, 3-hydroxydecanoate and 3-hydroxydodecanoate with monomer composition largely dominated by the C₈ (45.4 mol%) and C₁₀ (41.8 mol%) monomers. Oligomerisation of the mcl-PHA was achieved in mild conditions by acid hydrolysis which resulted in hydrolysed mcl-PHA with a molecular weight of around 10 kDa.

The DSC based analysis did not show any sign of oligo-HA crystallization as a separate phase in blends with oligo-HA content up to 20wt%. This implied good compatibility of the oligo-HA with P(3HB) in the amorphous phase and plasticisation saturation did not occur in the whole range blend compositions.

Although the addition of oligo-HA to P(3HB) resulted in only a small decrease in melting temperature, it led to a significant drop in the crystallinity of P(3HB). Thus, the amorphous phase presented a larger fraction in blends compared with that of neat P(3HB), which increased with an increase in oligo-HA content. This influenced the kinetics of maturation of the P(3HB) crystalline phase. The crystallinity defined by DSC did not change for the blends after two-weeks of storage at ambient temperature, while the crystallinity of neat P(3HB) increased during storage of up to 7 weeks. This implied that the larger fraction of the more mobile amorphous phase facilitated P(3HB) crystallization in blends, however, the overall degree of crystallinity reduced in blends.

Mechanical properties of both neat P(3HB) and P(3HB)/oligo-HA blends gradually changed during the 7-week ageing. Considering that P(3HB) crystallization in blends completed in two weeks, the changes in mechanical properties after two weeks must be due to the physical ageing of the amorphous
phase. Although the addition of oligo-HA did not eliminate the increase in stiffness of P(3HB)-based materials attributed to the ageing processes, the oligo-HA had a clear plasticizing effect on P(3HB) resulting in softer, more pliable materials. A threefold decrease in stiffness was achieved for the blend containing 20 wt% of oligo-HA and this material could withstand 20% deformation at break, resulting in a completely new modified form of P(3HB) with both high stiffness and good ductility. Such materials can replace the scl-mcl PHA copolymer in applications which require both these properties.

Lower stiffness of the blend materials correlates with the decreased crystallinity. Moreover, it is known that 2/3rd of the amorphous phase in P(3HB) is in a rigid state, which is a vitrified amorphous material [49]. The addition of oligo-HA most likely improves the mobility of the amorphous phase due to higher mobility of oligo-HA and hence resulted in a decrease of the rigid amorphous fraction. The fraction of rigid amorphous phase might change non-monotonically with oligo-HA content which could be a reason for the higher strength and stiffness of the 90/10 blend compared with the 95/5 blend. Another reason might be the difference in crystallite sizes for these blends. However, such an effect of relatively small amounts of oligo-HA on the mechanical properties of the aged samples requires further investigation. The excellent plasticizing effect of the oligo-HAs enabled the production of P(3HB)-based materials with a wider range of mechanical properties, thereby increasing the potential of their applications in soft tissue engineering and other applications where the balance of stiffness and ductility is essential.

The C2C12 mouse myoblast cell line was used as an in vitro model for attachment and proliferation of cells relevant to skeletal muscle and cardiac tissue engineering. It was proven that the novel P(3HB)-based blend materials were not cytotoxic. The C2C12 cells attached well and grew on the surface of the films exhibiting philopodia like structures. All the P(3HB)-based blend materials showed a much better ability to support cell growth compared with the control TCP. Further, cell proliferation on the surface of the blends improved in comparison with neat P(3HB) and increased with the
increase in oligo-HA content. The C2C12 cells on the blends also exhibited better elongation and tendency to achieve confluence, an indication of the initial differentiation of the cells to form functional muscle, as compared to the neat P(3HB) films [50]. Also, the increasing plasticizer content resulted in progressively higher surface roughness but decreased the wettability of blend films. Thus, the improvement of cell attachment and proliferation were induced by the topological and chemical cues. It was shown that protein adsorption increased with the increase in the concentration of the oligo-HA fraction in the blend. Protein adsorption is a preceding step before cells attach and spread over the surface [48]. The improved affinity of blends towards proteins contributed to the materials’ enhanced ability to support cell proliferation. Also, by variation of the plasticizer content mechanical properties of the blend were changed which enables variations in the biomechanical environment of the films. Mechanobiology is an emerging area of science where the role of substrate mechanics in the behaviour and functions of individual cells is being investigated [51]. In [52] a variation in the substrate stiffness modulated adhesion, proliferation and differentiation of C2C12 cells with stiffer surfaces supporting better adhesion and proliferation of C2C12 cells. In our study C2C12 proliferation improved with the decrease in the material stiffness. However, the stiffest substrate (Young’s modulus 382 kPa) used in [52] was softer than the softest 80/20 blend (Young’s modulus 480 MPa). Thus, materials developed in this study further extended the range of substrate stiffness available for soft tissue engineering. The observed enhancement of C2C12 cell proliferation on the relatively softer blends was most likely due to better mimicking of the physiological biomechanical environment of the myoblasts.

5. Conclusions

Further progress in tissue engineering and regenerative medicine requires a variety of biomaterials with different mechanical properties, biodegradability profiles and biological response. This study has focused on the approach of formulating binary systems based on an inherently stiff and brittle polymer, P(3HB), to achieve softer, more pliable biodegradable biomaterials. Additionally, this work
presents novel results involving new developments in microbial production of PHAs. P(3HB) was synthesised using *B. subtilis* OK2, a bacterial strain relatively unexplored in the production of scl-PHAs. Furthermore, as a way of improving the cost efficiency of PHA production, the mcl-PHA copolymer including 3-hydroxyhexanoate, 3-hydroxyoctanoate, 3-hydroxydecanoate and 3-hydroxydodecanoate was successfully synthesised, for the first time, using *P. mendocina* CH50 with waste frying oil as the sole carbon source. An oligomeric derivative of this elastomeric copolymer was produced by acid hydrolysis and explored as a unique biodegradable and biocompatible plasticizer for P(3HB). Characterisation of the thermal and mechanical properties of P(3HB)/oligo-HA blends demonstrated the plasticising effect of the oligo-HAs on P(3HB). Plasticising of P(3HB) with oligo-HA resulted in softer and more pliable biomaterials based on P(3HB), with a huge potential for applications in soft tissue engineering and other medical applications. This approach can be extended to all PHA biomaterials which could be used as biologically safe, biodegradable, non-migrating plasticizers.

In order to demonstrate the applicability of such blends as materials suited for soft tissue engineering, biocompatibility was tested with the C2C12 mouse myoblast cell line. The P(3HB)/oligo-HA blends were not cytotoxic and showed enhanced cell viability and proliferation with an increase in the oligo-HA content. Surface hydrophobicity and roughness also increased with the increase in the content of oligo-HA in the blends. Such physicochemical and topological changes resulted in the enhancement of surface affinity towards proteins. All these factors defined the improved ability of the blends to support cell proliferation. Also, the elastic modulus of the blends progressively decreased with the growing content of oligo-HA, which suggested that the mechanical cue is also an important factor responsible for improved cell proliferation on the blends. The plasticising of P(3HB) with oligomeric mcl-PHAs widens the range of mechanical properties of materials based on P(3HB) and hence offers biodegradable materials with good biocompatibility and great potential in regenerative medicine. This approach has further implications for other applications such as biodegradable coronary artery
stents, nerve guidance conduits and drug delivery since it mitigates P(3HB) embrittlement and results in more elastomeric P(3HB) based biomaterials with reasonable stiffness, a great step forward in the future applications of this well-established biomaterial.

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