

# The homopolymer, poly(3-hydroxyoctanoate), P(3HO), as a matrix material for soft tissue engineering

Ranjana Rai<sup>a</sup>, Aldo R. Boccaccini<sup>b,c</sup>, Jonathan C. Knowles<sup>d,e</sup>,  
Nicola Mordon<sup>d</sup>, Vehid Salih<sup>d</sup>, Ian C. Locke<sup>f</sup>, M. Moshrefi-Torbati<sup>g</sup>,  
Tajalli Keshavarz<sup>a</sup> and Ipsita Roy<sup>a\*</sup>

<sup>a</sup>*Department of Molecular and Applied Bio Sciences, School of Life Sciences, University of Westminster,  
London W1W 6UW, UK*

<sup>b</sup>*Department of Materials, Imperial College London, London SW7 2AZ, UK*

<sup>c</sup>*Department of Materials Science and Engineering, University of Erlangen – Nuremberg Cauestr. 6., 91058  
Erlangen, Germany.*

<sup>d</sup>*Division of Biomaterials and Tissue Engineering, UCL Eastman Dental Institute, London WC1X 8LD, UK*

<sup>e</sup>*WCU Research Centre of Nanobiomedical Science, Dankook University, San#29, Anseo-dong,  
Dongnam-gu, Cheonan-si, Chungnam, 330-714, South Korea*

<sup>f</sup>*Department of Biomedical Sciences, School of Life Sciences, University of Westminster,  
London W1W 6UW, UK*

<sup>g</sup>*School of Engineering Sciences, University of Southampton, Southampton SO171BJ, UK*

## Abstract

The homopolymer poly(3-hydroxyoctanoate), P(3HO) produced from *Pseudomonas mendocina* using octanoate as carbon feed was studied as a potential biomaterial for soft tissue engineering i.e. as cardiac patch and as matrices for skin tissue engineering. The polymer was fabricated into neat solvent cast films of 5 and 10 wt %. Microstructural studies revealed the films as having smooth surface topography with a root mean square (RMS) value of 0.238  $\mu\text{m}$ . The films also possessed moderate hydrophilicity when compared to other monomers of the PHA family. Stress-strain curves of the films obtained was typical of that of elastomeric polymers. This elastomeric and flexible nature of the films makes them promising candidates for the proposed applications. Biocompatibility studies using the HaCaT keratinocyte cell line showed that the films were able to support the attachment, differentiation and maturation of the HaCaT cells. *In vitro* degradation studies over a period of 4 months showed that the water absorption and weight loss increased progressively with time for the films. The films underwent hydrolytic degradation initiated on the surface and also showed an ageing effect.

**Keywords:** Polyhydroxyalkanoate, biopolymer, *P. mendocina*, soft tissue engineering and cardiac patch.

## INTRODUCTION

In the early 1990s a paradigm shift occurred in medicine from using synthetic and tissue grafts to a tissue engineering approach in order to address limitations of tissue grafting and alloplastic tissue repair <sup>[1, 2]</sup>. The approach is based on the fundamental notion that the body is able to heal itself <sup>[3]</sup> and involves the complex interactions between cells genes and/or numerous proteins within a porous degradable matrix material known as scaffold <sup>[2]</sup>. The scaffold or matrix material, far from being a passive component, plays an important role in tissue regeneration by preserving tissue volume, delivering biofactors and providing temporary mechanical support <sup>[1, 2]</sup>. An ideal scaffold must be biocompatible, balance mechanical function with tissue delivery and have degradability tailored to match tissue regenerative rate.

Numerous studies have been carried out on synthetic, natural and a combination of natural and synthetic materials for their use as scaffold/matrix for a wide ranging tissue engineering application <sup>[4, 5]</sup>. One such family of natural materials (biopolymers) attracting great interest as a scaffold material are the polyhydroxyalkanoates, PHAs. PHAs are polyesters of 3-hydroxyacids biosynthesized by numerous Gram positive and Gram negative bacteria under nutrient(s) limiting conditions like nitrogen, potassium, sulphur, magnesium and phosphate, in the presence of excess carbon source <sup>[6, 7]</sup>. These are accumulated as intracellular carbon and energy storage granules by fermentation of renewable resources. The PHAs may be short chain length (scl) containing C<sub>3</sub> to C<sub>5</sub> carbon atoms or medium chain length (mcl) containing C<sub>6</sub> to C<sub>14</sub> carbon atoms. Once extracted, PHAs exhibit properties ranging from hard and brittle to soft and elastomeric in nature. Scl-PHAs like poly(3-hydroxybutyrate), P(3HB), are hard and brittle in contrast to mcl-PHAs and their copolymers like poly(3-hydroxyhexanoate-co-3-hydroxyoctanoate), P(3HHx-co-3HO) that are soft and elastomeric. In addition to these wide ranging physical properties exhibited by the PHAs, this family of biopolyester are also biodegradable, bioresorbable and biocompatible. Owing to these amenable properties numerous studies have been carried out using PHAs as scaffold for hard and soft tissue engineering. The hard and brittle scl-PHA like P(3HB) has been studied for hard tissue engineering such as bone and the soft and elastomeric PHAs like P(3HHx-co-3HO) have been studied for soft tissue engineering such as heart valves, other vascular applications, skin tissue engineering and wound healing applications <sup>[5, 8]</sup>.

We have successfully produced a homopolymer, poly(3-hydroxyoctanoate), P(3HO), from *P. mendocina* using octanoate as the feed. Detailed structural, mechanical, thermal and molecular weight analyses of this polymer have been carried out <sup>[9]</sup>. In this work we describe, for the first time, the fabrication of the P(3HO) homopolymer into neat two dimensional films. A detailed microstructural, thermal, mechanical, *in vitro* degradation and biocompatibility assessment of the films has been carried out in order to assess the potential of P(3HO) as a matrix material for soft tissue engineering such as cardiac patch application and as support material for skin tissue engineering.

Myocardial infarction is one of the major causes of death in patients suffering from cardiovascular diseases (CVD). Treatment to date has relied on heart transplantation and the use of ventricular assisted devices (VAD). However, lack of organ donors and high cost of VADs have presented serious limitations. An alternative approach is to deliver cardiac cells to the heart by using a tissue engineering strategy; here a biodegradable patch would be populated, *in vitro*, with cardiac cells and then implanted onto the infarct region <sup>[10]</sup>. In this study the P(3HO) neat film is proposed to be used as a cardiac patch material. The cardiac patch will serve two functions, delivery of healthy cardiac cells into the infarct region and to provide left ventricular (LV) restraint, since myocardial infarction can impair overall pump function of the left ventricle leading to congestive heart failure.

Also, in the context of skin tissue engineering, another possible application of P(3HO) 2D films is to use it as matrices for engineering skin tissues which could be used for wound healing. The human skin is the largest organ of the body. The skin protects the body from the external environment by maintaining temperature and haemostasis in addition to performing sensory detection. Skin tissue engineering is very important as it is needed to provide skin grafts to permanently replace damaged or missing skin or to provide a temporary wound covering to burn victims, also for non healing wounds such as diabetic ulcers, venous ulcers and cosmetic surgery. Damaged skin or a non healing wound could compromise the health of an individual <sup>[11]</sup>. Much research has been carried out on the development and clinical use of tissue engineered skin. Skin cells have been seeded and populated into suitable films. The skin cell film construct is then grafted to the wound; cells then proliferate from the film to the wound bed forming cell clusters and ultimately the normal epidermis. The film in addition to supplying healthy cells also provides protection to the wound until it is degraded or absorbed <sup>[12, 13]</sup>.

## **EXPERIMENTAL PART**

### **Fabrication of films:**

The homopolymer P(3HO) was produced from *P. mendocina* using octanoate as described elsewhere <sup>[9]</sup>. Neat P(3HO) films were fabricated using the solvent casting method. The films were fabricated by using 5 and 10 % of the polymer in 10 mL of CHCl<sub>3</sub>. The polymer was well dissolved after which the polymer solution was filtered and the films made by casting the polymer solution into glass petri dishes. The solution was then left to air dry at room temperature for 1 week followed by freeze drying for 10 days.

### **Microstructural studies:**

#### **Scanning electron microscopy (SEM)**

Microstructural studies for the surface topography were also carried out using a JEOL 5610LV scanning electron microscope. The samples were placed on 8 mm diameter aluminium stubs and then sputter coated with gold using an EMITECH-K550 sputtering device. The operating pressure of  $7 \times 10^{-2}$  bar and deposition current of 20 mA for 2 minutes were used. The SEM images were taken with an acceleration voltage of 15 kV (maximum) to avoid incineration of the polymer due to the beam heat.

#### **White light interferometry study (Zygo®)**

White light interferometry was used to obtain 3D images of the surface topography of samples by means of the analyzer Zygo® (New View 200 OMP 0407C). This measurement allowed the quantification of the roughness and investigation of the topography of the surfaces.

#### **Contact angle study**

Static contact angle measurements were carried out to evaluate the wettability i.e. hydrophilicity of the fabricated films. A gas tight microsyringe was used to place an equal volume of water (<10 µl) on every sample by means of forming a drop. Photos (frame interval of 1 second, number of frames = 100) were taken to record the shape of the drops. The water contact angles on the specimens were measured by analysing the recorded drop images using the Windows based KSV CAM software. Six repeats for each sample were

carried out. The experiment was done on a KSV CAM 200 optical contact angle meter (KSV Instruments Ltd).

### **Mechanical properties**

Tensile testing was carried out using a Perkin–Elmer dynamic mechanical analyzer at room temperature. The test was carried out on polymer strips of 10 mm length and 4 mm width, cut from the solvent cast polymer films. The initial load was set to 1 mN and then increased to 6000 mN at the rate of 200 mN min<sup>-1</sup>. The test was carried out on 6 repeats of the samples. Young's modulus, stress and strain were recorded during the test. The Young's modulus was calculated from the initial strain range as described in the literature <sup>[14]</sup> i.e. when the polymer had strained up to 15%.

### **Thermal properties**

The thermal properties of the polymer, i.e. glass transition temperature ( $T_g$ ) and melting temperature ( $T_m$ ) were measured by carrying out differential scanning calorimetry (DSC) using a Perkin Elmer Pyris Diamond DSC (Perkin Elmer Instrument). The amount of polymer used for the study ranged from 8 to 10 mg and the sample was encapsulated in standard aluminium pans. All tests were carried out under inert nitrogen. The samples were heated/cooled/heated at a heating rate of 20°C min<sup>-1</sup> between -57°C and 100°C. The test was carried out on 9 repeats of the samples.

### ***In vitro* degradation studies**

*In vitro* degradation studies of the fabricated 2D films were carried out. The degradation behaviour was studied for a period of 1, 2 and 4 months in phosphate buffer saline (PBS), Dulbecco's Modified Eagles Medium (DMEM) and Dulbecco's Modified Eagles Medium Knock Out (DMEM<sup>KT</sup>).

PBS was chosen since it is one of the buffer systems that regulate the acid or base balance in the body. DMEM medium was chosen since the biocompatibility studies for the fabricated films were carried out using the HaCaT cell line and DMEM was the medium chosen to culture these cells. Similarly, DMEM<sup>KT</sup> medium was also chosen since for pericardial patch application, the films would be seeded with embryonic stem cells and DMEM<sup>KT</sup> is one of the most optimum culture media types that support the growth of these cells.

### **Water uptake, weight loss and pH measurements**

The degradation kinetics was determined by measuring the % water uptake or absorption (% WA) and % weight loss (% WL). For these, all the samples were first weighed to obtain the dry weight  $M_0$  ( $M_0$ , the initial weight of the sample), immersed in the respective media and kept under static conditions, at 37°C, until no further weight loss was evident. The medium was changed once a week. At each prescheduled incubation time point the films were collected and analysed for water absorption (% WA) and weight loss (% WL) behaviour. For measuring the % WA of the samples, the immersed samples were removed at given time points, the surface was gently wiped with a tissue paper and the weight was measured  $M_w$ , wet ( $M_w$ , the weight of the samples after immersion in the medium). Similarly, for measuring the % WL, the samples were withdrawn from the medium, washed several times with deionised water and dried at 37°C overnight and subsequently weighed  $M_t$ , dry ( $M_t$ , the dry weight of the samples after immersion in the media followed by drying). Water absorption and weight loss were calculated using the following equations:

$$\% \text{ WA} = [(M_w \text{ wet} - M_t \text{ dry}) / M_t \text{ dry}] 100$$

$$\% \text{ WL} = [(M_0 \text{ dry} - M_t \text{ dry}) / M_0 \text{ dry}] 100$$

The medium in which the films were incubated were changed every week. The pH changes of the media were then measured at the end of each incubation time point at ambient conditions.

### **Mechanical properties**

The *in vitro* degraded films were studied for their mechanical properties and surface degradation using SEM as described above.

### **Protein adsorption studies**

Protein adsorption assay was performed using foetal bovine serum, (FBS). Square films, (1 cm<sup>2</sup> in area), were incubated in 400 µl of undiluted FBS, at 37°C, for 24 hrs. After incubation, the samples were rinsed with PBS, thrice. The samples were then incubated in 1 mL of 2% sodium dodecyl sulfate (SDS) in PBS for 24 hrs at room temperature and under vigorous shaking to further collect the adsorbed proteins. The amount of total protein adsorbed on the surface of the samples was quantified using a commercial protein quantification kit (Pierce, Rockford, IL). The optical density of the samples was measured spectrophotometrically at 562 nm against a calibration curve using bovine serum albumin as

per the manufacturer's protocol. The samples incubated in PBS were used as a negative control. The assay was carried out in triplicates.

## ***In vitro* cytocompatibility study**

### **Cell culture study**

The *in vitro* cell culture studies were carried out on the P(3HO) neat films using the Human keratinocyte cell line, the HaCaT cell line, which was cultured in DMEM growth medium, supplemented with 10% foetal calf serum, 1% glutamine and 1% penicillin and streptomycin solution. The penicillin and streptomycin used was one solution. The cells were incubated at 37°C in a 5% CO<sub>2</sub> humidified atmosphere and culture medium changed every 2 days.

### **Cell seeding on the test samples**

Polymer 2D films, 1 cm<sup>2</sup> in area (square strips), were UV sterilised for 30 minutes on each side and passivated in culture medium for 12 h. A cell density of 20,000 cells was used to seed the scaffolds, kept in 24 well plates. The cells were analysed after 1, 4 and 7 days for cell adhesion, proliferation and morphology. Cell culture studies were carried out on triplicate samples per experiment.

### **Cell adhesion, proliferation and morphology**

Cell adhesion and proliferation studies were carried out using the Neutral Red (NR) assay as described elsewhere<sup>[15]</sup>. The total NR uptake was used as a measure of the cell's proliferation and viability (% NR uptake is directly proportional to the number of live and uninjured cells) and was compared to a control population, grown on tissue culture plates. The control was normalised to 100%.

### **SEM preparation for viewing of cells**

The P(3HO) films were examined under SEM in order to observe the HaCaT cell spreading and attachment on the surface of the samples. The specimens were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, for 12 h, at 4°C. Samples were dehydrated using graded series of alcohols. The samples were then left to air dry for 30 min. The dried samples were then attached to aluminium stubs, gold coated and examined for SEM as described earlier.

## RESULTS AND DISCUSSION

### Microstructural studies

The fabricated neat P(3HO) films were subjected to a series of analyses to assess their surface and microstructural properties. Surface topography, roughness and wettability have been reported to play a pivotal role in the applicability of a potential biomaterial. The surface study of the fabricated films (**Figure 1A**) using SEM (**Figure 1B**) revealed that the P(3HO) films had a smooth surface topography. Surface analysis of the films was also carried out by white light interferometry using a Zygo<sup>®</sup> instrument to visualise the topography of the films as shown in the surface scan in **Figure 1C**. White light interferometry analysis revealed a typical RMS value of 0.238  $\mu\text{m}$ . The film had a smooth surface as opposed to the hard and brittle P(3HB) having a RMS value of 2.01  $\mu\text{m}$ .

Water contact angle studies of the fabricated films were also carried out to assess their wettability, i.e. hydrophilicity. Surfaces with  $\theta_{\text{H}_2\text{O}}$  less than  $70^\circ$  is considered to be hydrophilic and  $\theta_{\text{H}_2\text{O}}$  greater than  $70^\circ$  as hydrophobic<sup>[13]</sup>. Static contact angle measurements of the fabricated films are given in **Table 1**. For comparison, the contact angle values measured for other PHAs in literature are also included in the table. Therefore, in this study the fabricated neat P(3HO) with  $\theta_{\text{H}_2\text{O}} = 77.3^\circ \pm 1$  (5 wt%) and  $\theta_{\text{H}_2\text{O}} = 78^\circ \pm 1$  (10 wt%) were found to be more hydrophilic than most of the other PHAs, except for P(3HO) copolymer (containing 84.5 mol% C<sub>8</sub>, 6 mol% C<sub>6</sub> and 4.3 mol% C<sub>10</sub>) which had a  $\theta_{\text{H}_2\text{O}}$  of  $73.8^\circ$ <sup>[16]</sup>.

### Mechanical properties

In this study, no point of discontinuity in the slope of the stress-strain curve (commonly known as “knee”)<sup>[20]</sup> appeared for the 5 and 10 wt% films (**Figure 2**). The Young’s modulus values calculated from the slope of the curves were  $1.40 \pm 0.6$  MPa for the 5 wt% film, **Figure 2(A)**, and  $3.09 \pm 0.7$  MPa for the 10 wt% film, **Figure 2(B)**.

The static tensile studies therefore showed that the fabricated films, like other mcl-PHAs reported in literature, exhibited an elastomeric nature. Marchessault et al.<sup>[21]</sup> found the Young’s modulus ‘E’ value for P(3HO) to be 17 MPa and the % elongation values ranging between 250-350%<sup>[21]</sup>. For mcl-PHA containing 86% of 3HO and minor quantities of 3-hydroxydecanoate and 3-hydroxyhexanoate the ‘E’ value of the polymer film (1.6 mm thickness) was  $7.6 \pm 0.5$  MPa and % elongation to break of 380%<sup>[22]</sup>. Similarly Asrar et al.<sup>[23]</sup> observed Young’s modulus values ranging from 155 to 600 MPa and elongation to break

ranging between 6.5 to 43% for P(3HO) thermally processed films (micrometer thick, values not given) containing 2.5 to 9.5 mol % of 3-hydroxyhexanoate (3HHx). In studies carried out by Ouyang et al. <sup>[24]</sup> mcl-PHA solvent casted films (100 µm thick) containing different mol% of 3-hydroxydodecanoate, 3(HDD), i.e. 15, 28 and 39, had Young's modulus values of 3.6, 6.0 and 11.5 MPa respectively <sup>[24]</sup>. Thus, the Young's modulus values of the fabricated films in this present study were relatively low when compared to other values reported in literature. This low stiffness, (E value) of the films could be due to the homopolymeric nature of the P(3HO) films, which because of the long carbon backbone of the same type (all C<sub>8</sub> monomers) were able to deform easily to dissipate the applied stress. Thus, this study has shown that the fabricated P(3HO) films were less stiff than most other PHAs described in literature.

### **Thermal properties**

Thermal studies of the fabricated films were also carried out since thermal stability has an important implication on the material's processability and end goal application. The thermal properties of the films are depicted in **Figure 3(A-B)** and summarised in **Table 2**. During the first heat scan the polymer chains in the crystalline phase of the polymer has melted and become disordered. This absorption of energy for the melting of the polymer chains in the crystalline phase was reflected as the T<sub>m</sub> peak. Once melted, the polymer chains were unable to rearrange themselves into ordered structures during the cooling run following the first heating run. This inability of the melted polymer chains to rearrange themselves into ordered crystalline lattices, led to the absence of a T<sub>m</sub> peak, representing the transition of the crystalline phase into the amorphous state during the second heat scan.

### ***In vitro* degradation study**

The degradation behaviour of a matrix material is of paramount importance and greatly affects its potential as a biomaterial. *In vitro* degradation of the films was carried out in order to understand how these films degrade and how their physical and chemical properties change with degradation.

Water absorption (%WA) and weight loss (%WL) behaviour of the films were studied over the incubation time period of 4 months. The results, **Figure 4(A-B)**, showed that the films absorbed water and lost weight during the incubation period, thus indicating that the films

were undergoing degradation. Both the water absorption and the weight loss increased progressively with time in all the three media i.e. PBS, DMEM and DMEM<sup>KT</sup>.

In a study carried out on the degradation of poly(3-hydroxybutyrate-co-3-hydroxyvalerate), P(3HB-co-3HV), Holland et al. <sup>[25]</sup> observed water absorption in polymers during a 120 day experiment and found that increased water absorption was associated with progressive degradation of the samples. As the films in this study were only incubated in enzyme free media, the degradation of the films must have occurred due to abiotic non enzymatic hydrolysis. The water molecules react with the polymer, cleaving the ester bond, thus exposing the carboxylic acid group and the hydroxyl group. The degradation of the films commenced at the surface, as observed in the SEM images (**Figure 5(A1-A2)**), which shows degraded polymer flakes on the surface of the films. This is because unlike polymers like PLGA which is an amorphous polymer with water absorption resulting in bulk degradation, in P(3HO), a semi crystalline polymer, degradation is multiphasic like that observed with the broader PHA family.

In the first stage, spanning a few weeks, the amorphous phase of the polymer begins to degrade. This is because the crystalline regions of the polymer are impermeable to water, hydrolysis is therefore restricted to the amorphous regions of the polymer and to the fringes of the crystalline region <sup>[26]</sup>. Next, the crystalline part of the polymer begins to degrade resulting in the formation of monomers, dimers and tetramers; simultaneously the molecular mass also decreases. Progressively with time, the degradation process develops and the polymer loses its mass <sup>[27]</sup>. Such hydrolytic degradation of PHAs have been previously described in literature and is known to be a slow process when compared to the enzymatic hydrolysis of PHAs <sup>[28]</sup>. This is because of the relatively high crystallinity of PHAs which leads to increased impermeability of water into the crystalline regions <sup>[26]</sup> and also due to the hydrophobic nature of long alkyl pendant chains <sup>[29]</sup>. This slow hydrolytic degradation could be a reason why the fabricated films had lost only 15% of their weight by the end of the degradation studies. However, the water absorption and weight loss observed in this study for the neat films were higher than those observed for other PHAs, also studied for hydrolytic degradation. For instance, no significant weight loss was observed when P(3HB), P(3HB-co-3HV) samples were incubated for 180 days, at 37°C, in aqueous media <sup>[30, 31]</sup>. Marios et al. <sup>[28]</sup> studied the *in vitro* degradation of the P(3HO) film containing 3 mol% 3HHx in water and phosphate buffer saline. The films showed negligible mass loss of less than 1% after 24 months of incubation <sup>[28]</sup>. Numerous factors affect the biodegradability of PHAs such as

stereoregularity, molecular mass, monomeric composition and crystallinity of the polymer. Studies carried out by Mochizuki <sup>[32]</sup> and Tokiwa and Calabia <sup>[33]</sup> showed that biodegradation of PHAs is influenced by the chemical structure (i.e. the presence of functional groups in the polymer chain, hydrophilicity / hydrophobicity balance) and presence of ordered structure such as crystallinity, orientation and morphological properties <sup>[32, 33]</sup>. Usually the degradation of the polymer decreases with the increase of highly ordered structure i.e. increased crystallinity. Since the P(3HO) used in this study has lower crystallinity than P(3HB) and P(3HB-co-3HV) <sup>[30, 31]</sup> the fabricated P(3HO) films could be expected to be more degradable than P(3HB) and P(3HB-co-3HV), described in literature.

In this present study, the pH of all the three media, PBS, DMEM and DMEM<sup>KT</sup>, in which the neat films were incubated, increased progressively with time (**Figure 6**). The degradation of the P(3HO) films would result in the production of 3-hydroxyoctanoate or 3-hydroxyoctanoic acid depending on the pH of the media. If hydroxyoctanoic acid is denoted as HA, then its dissociation upon release after degradation can be represented as:



The pKa for 3-hydroxyoctanoic acid is 4.89. Since the pH of all the three media was 7.4, which is greater than the pKa of the acid (4.89), most of the acid molecules would exist in the form of 3-hydroxyoctanoate, a base, leading to an increase in the pH of the media in which the films were incubated.

When mechanical testing of the degraded films was carried out it was found that the Young's modulus of the polymer had increased after two months of incubation and again showed a reduction of the modulus after 4 months of incubation, as shown in **Figure 7**. Although the Young's modulus had decreased at 4 months, the value was still higher than those observed for the undegraded films. This increase in the Young's modulus of the films after degradation could be due to the ageing of the polymer <sup>[34]</sup>. The ageing in PHAs occurs due to the secondary crystallisation of the PHAs which involves the development of the interlamellar secondary crystals in the amorphous region of the semi crystalline polymer. The small crystallites produced restrict the motion of the polymer chains in the amorphous regions, thereby reducing the mobility of the polymer chain segments; thus raising the modulus and increasing the brittle nature of the material <sup>[35]</sup>. Many PHAs have been reported to show ageing behaviour <sup>[23, 35-37]</sup>. For example, when Asrar et al. <sup>[23]</sup> aged the copolymer

P(3HB-co-8.1 mol% 3HHx) for 11 days at room temperature, the Young's modulus increased from 18.8 MPa to 22.8 MPa. Alata et al. [36] studied the ageing effect on the mechanical properties of P(3HB-co-3HHx) containing different mol % of 3HHx, ranging from 5 to 18 mol%. After 60 days of ageing they found that the % elongation to break had decreased and the tensile strength had increased for all the copolymers. Similar observations were made for P(3HB) and copolymer of P(3HB-co-3HV) [35].

This ageing effect exhibited by the films in this present study is not desirable. This could be overcome by incorporating suitable hydrophilic polymers or plasticizers in the films. Such addition could also be employed for tailoring the degradation rate of the films, as addition of amorphous or hydrophilic additives lead to higher water absorption and accelerate hydrolysis. For example, the water content was found to be higher in P(3HB)/P(DL-lactic acid) than in P(3HB)/polycaprolactone [38].

In tissue engineering, it is important that the effect of the degradation products on cellular behaviour is considered for a comprehensive biocompatibility evaluation of the implant polymers. Studies were carried out by Sun and his group in 2007 [39], on the cellular responses of mouse fibroblast cell line L929 to the PHA degradation products, i.e. oligo-hydroxyalkanoates (OHAs). They found that the cytotoxicity of OHAs decreased with increasing OHA side chain length, thus indicating that medium chain length OHAs containing PHA, such as poly(3-hydroxybutyrate-co-3-hydroxyhexanoate), P(3HB-co-3HHx) and mcl-PHAs are more biocompatible than scl-PHAs. Therefore, degradation products of the fabricated neat P(3HO) films in this study can also be expected to have decreased the cytotoxic effect on the seeded cells as compared to the scl-PHAs, an important requirement for a biomaterial.

### **Protein adsorption and *in vitro* cell biocompatibility**

Many studies have been carried out to understand the cell and material interfacial relationships, particularly related to protein adsorption. This is because most mammalian cells are anchorage dependent and need a biocompatible protein rich surface for attachment, differentiation and migration to form new tissue [40-42]. It has been shown that cell adhesion takes place in two different stages. The first stage consists of the adsorption of water and a layer of proteins that selectively adhere onto the biomaterial surface, mediated by the surface properties of the substrate [43]. The second stage involves cell adhesion onto the layer of proteins, which is a more complex process, mediated by the extracellular matrix (ECM)

proteins, cell membrane proteins, and cytoskeletal proteins [42, 44]. Protein adsorption can thus modulate cell adhesion and survival. Therefore, protein adsorption is important in evaluating a film for tissue engineering. The adsorption of proteins onto the fabricated films, in the present study, was carried out using the whole protein serum. The total proteins adsorbed onto the surface of the films were 75 and 83.17  $\mu\text{g}/\text{cm}^2$  for the 5 and 10 wt% films respectively.

The *in vitro* cell biocompatibility studies of the films were carried out by seeding HaCaT cells onto the films and studying its proliferation over a duration of 1, 4 and 7 days. The results (**Figure 8**) show that the cell proliferation increased progressively on all the films and at day 7, the proliferation was 58.42% (5 wt% film) and 73.09% (10 wt% film) of the control. Keratinocytes are known to form four distinct layers and divide and differentiate as they move from the deeper layer to the outermost layers. This arrangement of cell layers from the bottom to the outermost is as follows: (1) stratum basale (basal layer) (2) stratum spinulosum (spiny or prickle cell layer), (3) stratum granulosum (granular layer) and (4) stratum corneum (horn sheet layer) [45]. SEM scans (**Figure 9**) of the cells also confirmed that the polymer matrix was able to support cell growth, differentiate and mature successfully into horn sheets at day 7.

## **Properties of films and their suitability for the proposed applications**

### **Poly-3-hydroxyoctanoate, P(3HO) neat film as a potential biomaterial for cardiac patch use**

The flexible and elastomeric nature of the films make them potentially suitable as biomaterials for heart patch application based on their stiffness, i.e. Young's modulus values. **Table 3** compares the properties of the various biomaterials which have been used for cardiac tissue engineering with the fabricated 5 wt% P(3HO) film in this study. Other biomaterials being studied for the application seem to be either too stiff, such as poly glycolic acid, (PGA), poly(L-lactic acid), (PLLA) or poly(DL-lactide), (PDLLA), whose Young's modulus value range in GPa or are very soft like collagen gel whose Young's modulus value is in the range 0.002-0.022 MPa. The stiffness of the fabricated P(3HO) films, in particular the 5 wt% film, is comparable to the higher end stiffness value of polyglycerol sebacate, PGS, ranging from 0.04 to 1.2 MPa, which is a synthetic polymer being studied as material for patch applications [46, 47]. An interesting point is that the property of P(3HO) can be tailored by various methods such as grafting acrylamide and carboxyl ions using plasma treatment [48]

and by blending it with other biocompatible elastomeric polymers such as collagen, so that its mechanical properties can match either the stiffness of the heart muscle at the beginning of diastole (stiffness is 10–20 kPa) or the stiffness at the end of diastole (200–500 kPa) [46].

### **P(3HO) film as a matrix for skin tissue engineering**

Numerous studies have been carried out on a number of natural, synthetic and a combination of natural and synthetic materials for skin tissue engineering applications [58, 59]. Some of the PHA monomers have also been studied as matrices for skin tissues such as P(3HB), P(3HB-co-3HV), P(3HB-co-4HB), P(3HB-co-3HHx) [13, 18]. However, up to now, P(3HO) has never been studied in the context of skin tissue engineering. To the author's knowledge this is the first such study of a homopolymer of P(3HO) and its biocompatibility with HaCaT cells.

In spite of the fact that tissue engineered skin is now a reality, challenges are still faced in allowing cultured mass skin cells to attach securely, often to very challenging wound beds. The stability of keratinocyte attachment to the underlying dermis is also an important issue when delivering keratinocytes to deep burns and wound beds, especially if such burns and wound beds are located in difficult contours of the body. Therefore, in such a case having a flexible and elastomeric support would be ideal, hence P(3HO) films fabricated in this study being flexible and elastomeric would easily fit into such difficult contours of the body. However, the mechanical properties, the Young's modulus value and the tensile strength of the fabricated P(3HO) films were found to be lower when compared to human skin [60]. One possible approach of improving the mechanical properties of the films in order to match the properties of human skin would be to blend P(3HO) with other biocompatible and mechanically suitable material for skin such as chitosan, (CH), hyaluronic acid and or poly(4-hydroxybutyrate), P(4HB) [13]. Blends of P(4HB)/Ha and P(4HB)/CH have been found to have mechanical properties suitable for human skin. **Table 4** compares the properties of the various biomaterials which have been used for skin tissue engineering.

## **CONCLUSION**

The homopolymer P(3HO) produced from *P. mendocina* was fabricated into films and studied for its suitability as a potential material for cardiac patch application and skin regeneration. The films were studied for their surface, microstructural, physical, *in vitro*

degradation behaviour and biocompatibility properties. Mechanically the films were found to possess flexible and elastomeric nature, a requirement in the proposed areas of applications. Biocompatibility studies showed that the films were able to support cell attachment, differentiation and maturation of the seeded HaCaT cells. This initial data suggests that P(3HO) films may be a potential for soft tissue engineering.

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