The Homopolymer Poly(3-hydroxyoctanoate) as a Matrix Material for Soft Tissue Engineering

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ABSTRACT: The homopolymer poly(3-hydroxyoctanoate), produced from *Pseudomonas mendocina* with octanoate as a carbon feed, was studied as a potential biomaterial for soft tissue engineering, that is, as a 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 a smooth surface topography with a root mean square value of 0.238 μm. The films also possessed moderate hydrophilicity when compared to other monomers of the polyhydroxyalkanoate 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 with the human adult low calcium temperature keratinocytes (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 aging effect. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 122: 3606–3617, 2011

Key words: biopolymer; polyester; elastomer; biodegradable

INTRODUCTION

In the early 1990s, a paradigm shift occurred in medicine from the use of synthetic and tissue grafts to a tissue engineering approach to address limitations of tissue grafting and alloplastic tissue repair.[1,2] The approach is based on the fundamental notion that the body is able to heal itself[3] and involves complex interactions between cells, genes, and/or numerous proteins within a porous degradable matrix material known as *scaffold*.[2] 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.[1,2] An ideal scaffold must be biocompatible, balance mechanical function with tissue delivery, and have degradability tailored to match the 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 range of tissue engineering application.[4,5] One such family of natural materials (biopolymers) attracting great interest as a scaffold material are polyhydroxyalkanoates (PHAs). PHAs are polyesters of 3-hydroxyacids biosynthesized by numerous Gram-positive and Gram-negative bacteria under conditions limiting nutrients, such as nitrogen, potassium, sulfur, magnesium, and phosphate, in the presence of excess carbon source.[6,7] These are

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accumulated as intracellular carbon and energy storage granules by the fermentation of renewable resources. The PHAs may be short chain length (scl), containing C_3 to C_5 carbon atoms, or medium chain length (mcl), containing C_6 to C_{14} carbon atoms. Once extracted, PHAs exhibit properties ranging from hard and brittle to soft and elastomeric in nature. scl-PHAs, such as poly(3-hydroxybutyrate) [P(3HB)], are hard and brittle in contrast to mcl-PHAs and their copolymers, such as poly(3-hydroxyhexanoate-*co*-3-hydroxyoctanoate) [P(3HHx-*co*-3HO)], which are soft and elastomeric. In addition to these wide-ranging physical properties exhibited by PHAs, this family of biopolyester are also biodegradable, bioresorbable, and biocompatible. Because of these amenable properties, numerous studies have been carried out using PHAs as scaffold for hard and soft tissue engineering. The hard and brittle scl-PHAs, such as P(3HB), have been studied for hard tissue engineering, such as bone, and the soft and elastomeric PHAs, such as 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.[5,8]

We successfully produced a homopolymer, poly (3-hydroxyoctanoate) [P(3HO)], from *Pseudomonas mendocina* using octanoate as the feed. Detailed structural, mechanical, thermal, and molecular-weight analyses of this polymer were carried out.[9] In this work, we describe, for the first time, the fabrication of the P(3HO) homopolymer into neat two-dimensional (2D) films. A detailed microstructural, thermal, mechanical, *in vitro* degradation, and biocompatibility assessment of the films was carried out to assess the potential of P(3HO) as a matrix material for soft tissue engineering, such as cardiac-patch application and as a support material for skin tissue engineering.

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

Also, in the context of skin tissue engineering, another possible application of P(3HO) 2D films is to use them 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 hemostasis 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; it is also important for nonhealing wounds, such as diabetic ulcers, venous ulcers, and cosmetic surgery. Damaged skin or a nonhealing wound could compromise the health of an individual.[11] 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, normal epidermis. The film, in addition to supplying healthy cells, also provides protection to the wound until it is degraded or absorbed.[12,13]

EXPERIMENTAL

Fabrication of films

The homopolymer P(3HO) was produced from *P. mendocina* with octanoate, as described elsewhere.[9] Neat P(3HO) films were fabricated with the solvent-casting method. The films were fabricated with 5 and 10% polymer in 10 mL of $CHCl_3$. The polymer was well dissolved, after which the polymer solution was filtered, and the films were made by the casting of 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 of the surface topography were also carried out with a JEOL 5610LV scanning electron microscope (Peabody, MA). The samples were placed on 8-mm-diameter aluminum stubs and then sputter-coated with gold with an EMITECH-K550 sputtering device (Emitech Ltd. Ashford, Kent, UK). An operating pressure of 7×10^{-2} bar and a deposition current of 20 mA for 2 min 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 three-dimensional (3D) images of the surface topography of the samples by means of a Zygo analyzer

(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, that is, the 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 the formation of a drop. Photos (frame interval = 1 s, number of frames = 100) were taken to record the shapes of the drops. The water contact angles (θ_{H2O}'s) on the specimens were measured by analysis of the recorded drop images with 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 with a PerkinElmer dynamic mechanical analyzer at room temperature. The test was carried out on polymer strips 10 mm in length and 4 mm in width cut from the solvent-cast polymer films. The initial load was set to 1 mN and then increased to 6000 mN at a rate of 200 mN/min. The test was carried out on six repeats of the samples. The Young's modulus (E'), stress, and strain were recorded during the test. E' was calculated from the initial strain range, as described in the literature,[14] that is, when the polymer had strained up to 15%.

Thermal properties

The thermal properties of the polymer, that is, glass-transition temperature (T_g) and melting temperature (T_m), were measured by differential scanning calorimetry with a PerkinElmer Pyris Diamond differential scanning calorimeter. The amount of polymer used for the study ranged from 8 to 10 mg, and the sample was encapsulated in standard aluminum pans. All tests were carried out under inert nitrogen. The samples were heated/cooled/heated at a heating rate of 20°C/min between −57 and 100°C. The test was carried out on nine repeats of the samples.

In vitro degradation studies

In vitro degradation studies of the fabricated 2D films were carried out. The degradation behavior was studied for periods 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 ($DMEM^{KT}$).

PBS was chosen because it is one of the buffer systems that regulate the acid or base balance in the body. DMEM medium was chosen because the biocompatibility studies for the fabricated films were carried out with the HaCaT cell line and DMEM was the medium chosen to culture these cells. Similarly, $DMEM^{KT}$ medium was also chosen because, for pericardial-patch application, the films would be seeded with embryonic stem cells and $DMEM^{KT}$ 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 were determined by measurement of the percentage water uptake or absorption (% WA) and percentage weight loss (% WL). For these, all of the samples were first weighed to obtain the dry weight [the initial weight of the sample ($M_{0\ \mathrm{dry}}$)] 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 analyzed for % WA and % WL behavior. To measure % 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; $M_{w\mathrm{wet}}$ is the weight of the samples after immersion in the medium). Similarly, to measure % WL, the samples were withdrawn from the medium, washed several times with deionized water, dried at 37°C overnight, and subsequently weighed ($M_{t\ \mathrm{dry}}$, the dry weight of the samples after immersion in the media followed by drying). % WA and % WL were calculated with the following equations:

$$\%\mathrm{WA} = [(M_{w\,\mathrm{wet}} - M_{t\,\mathrm{dry}})/M_{t\,\mathrm{dry}}] \times 100$$

$$\%\mathrm{WA} = [(M_{0\,\mathrm{dry}} - M_{t\,\mathrm{dry}})/M_{0\,\mathrm{dry}}] \times 100$$

The medium in which the films were incubated was 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 with SEM, as described previously.

Protein adsorption studies

A protein adsorption assay was performed with fetal bovine serum. Square films (1 cm^2 in area) were incubated in 400 μL of undiluted fetal bovine serum

at 37°C for 24 h. After incubation, the samples were rinsed with PBS three times. The samples were then incubated in 1 mL of 2% sodium dodecyl sulfate in PBS for 24 h 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 with a commercial protein quantification kit (Pierce, Rockford, IL). The optical density of the samples was measured spectrophotometrically at 562 nm against a calibration curve with 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 triplicate.

In vitro cytocompatibility study

Cell culture study

The *in vitro* cell culture studies were carried out on the P(3HO) neat films with the human keratinocyte cell line, the HaCaT cell line, which was cultured in DMEM growth medium supplemented with 10% fetal calf serum, 1% glutamine, and 1% penicillin and streptomycin solution. The penicillin and streptomycin used were one solution. The cells were incubated at 37°C in a 5% CO_2 humidified atmosphere, and the culture medium was changed every 2 days.

Cell seeding on the test samples

Polymer 2D films, 1 cm^2 in area (square strips),were UV-sterilized for 30 min on each side and passivated in culture medium for 12 h. A cell density of 20,000 cells was used to seed the scaffolds, which were kept in 24-well plates. The cells were analyzed 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 with the neutral red (NR) assay, as described elsewhere.[15] The total NR uptake was used as a measure of the cell's proliferation and viability (percentage NR uptake was 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 normalized to 100%.

SEM preparation for viewing of cells

The P(3HO) films were examined under SEM 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. The samples were dehydrated with a graded series of alcohols. The samples were then left to air-dry for 30 min. The dried samples were then attached to aluminum stubs, gold-coated, and examined by 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 [Fig. 1(a)] using SEM [Fig. 1(b)] revealed that the P(3HO) films had a smooth surface topography. Surface analysis of the films was also carried out by white-light interferometry with a Zygo instrument to visualize the topography of the films, as shown in the surface scan in Figure 1(c). White-light interferometry analysis revealed a typical root mean square value of 0.238 μm. The film had a smooth surface, as opposed to the hard and brittle P(3HB), and had a root mean square value of 2.01 μm.

θ_{H2O} studies of the fabricated films were also carried out to assess their wettability, that is, their hydrophilicity. Surfaces with a θ_{H2O} of less than 70° were considered to be hydrophilic, and those with θ_{H2O} values greater than 70° were considered to be hydrophobic.[13] Static contact angle measurements of the fabricated films are given in Table I. 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 $\theta_{H2O} = 77.3 \pm 1°$ (5 wt %) and $\theta_{H2O} = 78 \pm 1°$ (10 wt %) were found to be more hydrophilic than most of the other PHAs, except for the P(3HO) copolymer (containing 84.5 mol % C_8, 6 mol % C_6, and 4.3 mol % C_{10}), which had a θ_{H2O} of 73.8°.[16]

Mechanical properties

In this study, no point of discontinuity in the slope of the stress–strain curve (commonly known as *knee*)[20] appeared for the 5 and 10 wt % films (Fig. 2). E' values calculated from the slope of the curves were 1.40 ± 0.6 MPa for the 5 wt % film [Fig. 2(a)] and 3.09 ± 0.7 MPa for the 10 wt % film [Fig. 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.[21] found the E' value for P(3HO) to be 17 MPa and the percentage elongation values to range between 250 and 350%.[21] For mcl-PHA containing 86% of 3-hydroxyoctanoate (3HO) and minor quantities of 3-hydroxydecanoate and 3-hydroxyhexanoate

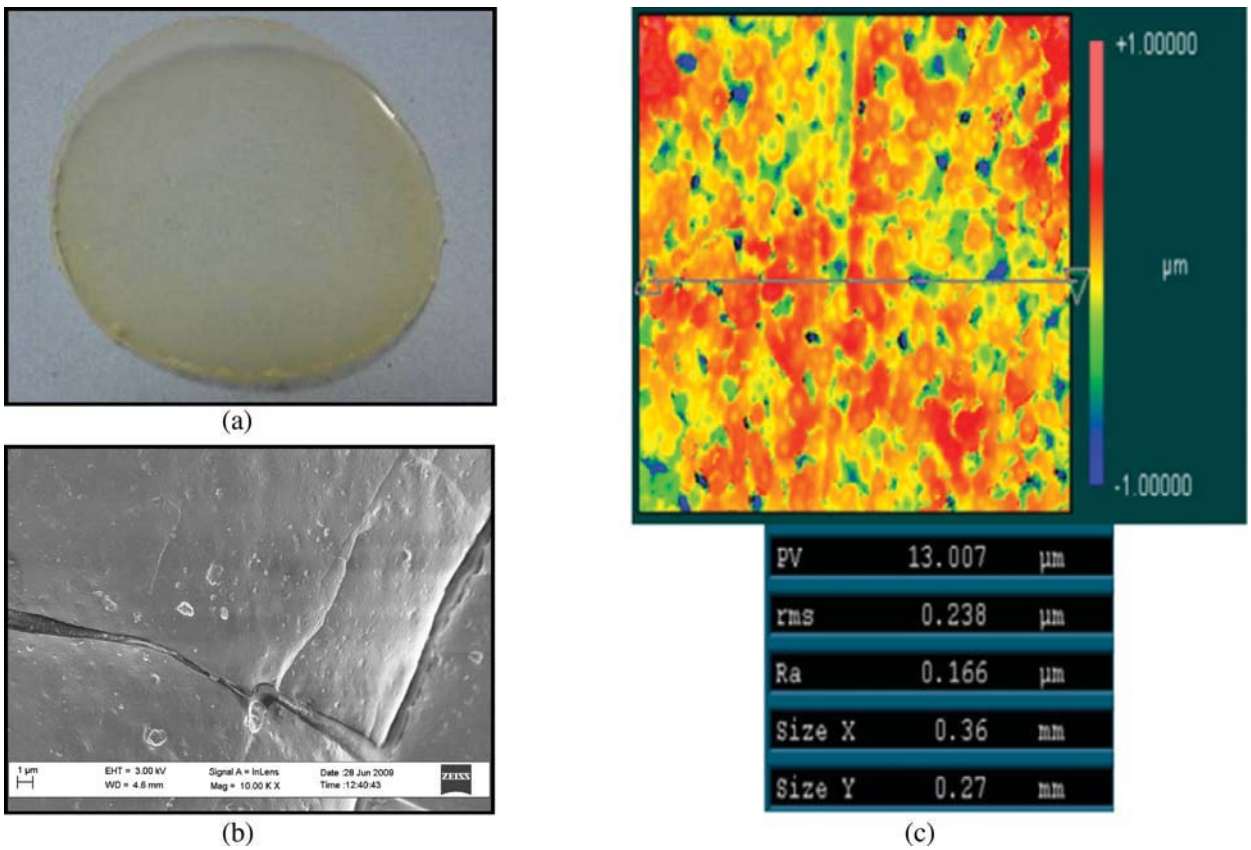


Figure 1 (a) Solvent-cast P(3HO) film, (b) cross section of the neat film, and (c) surface scan of the film with Zygo. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(3HHx), the E' value of the polymer film (1.6 mm thickness) was 7.6 ± 0.5 MPa, and the percentage elongation at break was 380%.[22] Similarly, Asrar et al.[23] observed E' values ranging from 155 to 600 MPa and elongation at break values ranging between 6.5 and 43% for P(3HO) thermally processed films (micrometer thick, values not given) containing 2.5–9.5 mol % 3HHx. In studies carried out by Ouyang et al.,[24] mcl-PHA solvent-cast films (100 µm thick) containing different molar percentages of 3-hydroxydodecanoate, that is, 15, 28, and 39, had E' values of 3.6, 6.0, and 11.5 MPa, respectively.[24] Thus, the E' values of the fabricated films in this study were relatively low compared to other values reported in literature. This low stiffness (E' value) of the films could have been due to the homopolymeric nature of the P(3HO) films, which, because of the long carbon backbone of the same type (all C_8 monomers), were able to deform easily to dissipate the applied stress. Thus, this study showed 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 because 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 summarized in Table II. During the first heat scan, the polymer chains in the crystalline phase of the polymer melted and became disordered. This absorption of energy for the melting of the polymer chains in the crystalline phase was reflected as the T_m peak. Once melted, the polymer chains were unable to rearrange themselves into ordered structures during the cooling run after the first heating run. This inability of the melted polymer chains to rearrange themselves

TABLE I
Comparison of the Contact Angle Values of the Fabricated Films in This Study with Those of PHA Monomers Reported in Literature

Polymer	θ_{H2O} (°)	Reference
P(3HO-*co*-3HHx-3HD)	73.8	16
P(3HO-*co*-3HU)	98	17
P(3HB-co3HHx)	85	18
P(3HB-*co*-3HV)	90	18
P(3HB)	87	19
P(3HO) (5 wt % film)	77.3	This study
P(3HO) (10 wt % film)	78	This study

P(3HO-*co*-3HU), poly(3-hydroxyoctanoate-*co*-3-hydroxy-10-undecenoate).

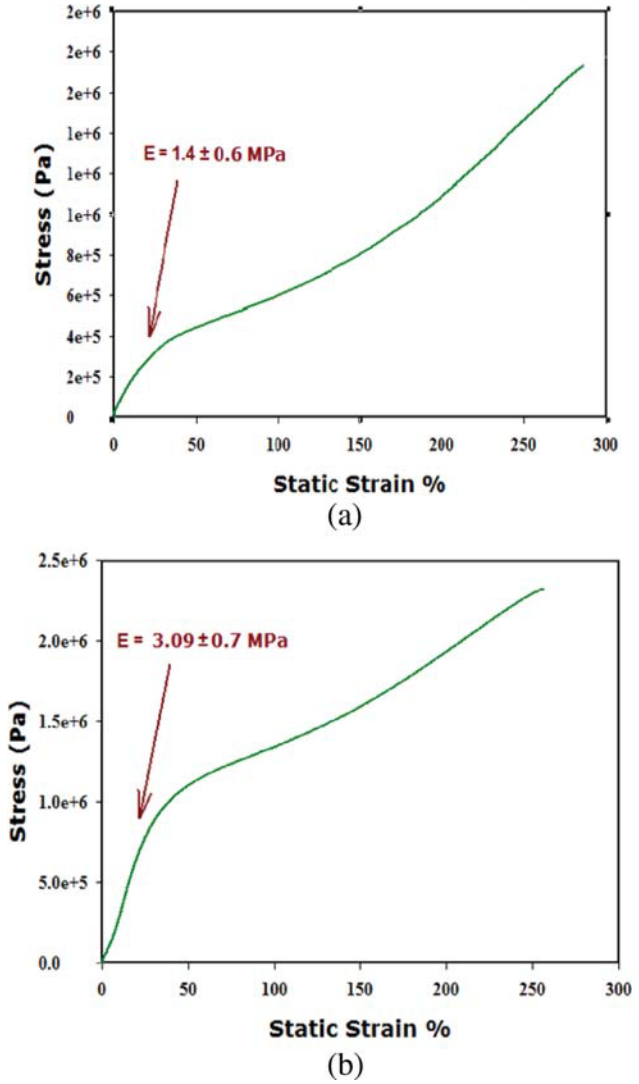


Figure 2 Stress–strain profile of the fabricated 5 and 10 wt % P(3HO) films. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

into ordered crystalline lattices led to the absence of a T_m peak, which represented the transition of the crystalline phase into the amorphous state during the second heat scan.

In vitro degradation study

The degradation behavior 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 to understand how these films degrade and how their physical and chemical properties change with degradation.

% WA and % WL behavior of the films was studied over an incubation time period of 4 months. The results, shown in Figure 4(a,b), show that the films absorbed water and lost weight during the incubation period and, thus, indicated that the films underwent degradation. Both % WA and % WL increased progressively with time in all three media, that is, PBS, DMEM, and DMEM^{KT}.

In a study carried out on the degradation of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)], Holland et al.[25] observed % WA in polymers during a 120-day experiment and found that increased % WA 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 because of abiotic nonenzymatic hydrolysis. The water molecules reacted with the polymer, cleaving the ester bond and, 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 [Fig. 5(a1,a2)], which show

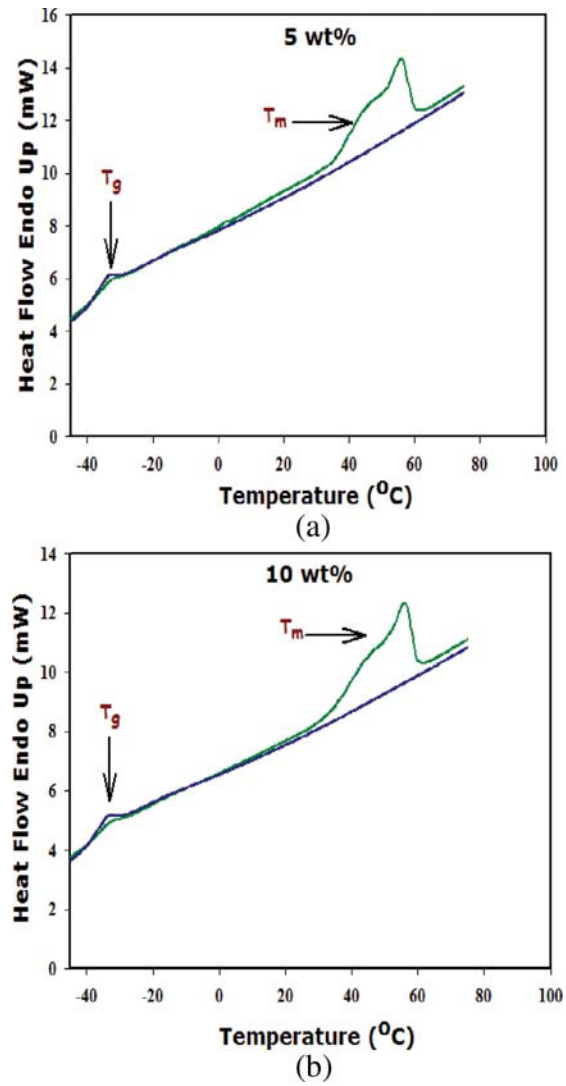


Figure 3 Thermal profile of the fabricated films: (a) 5 and (b) 10 wt % film showing T_g and T_m. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE II
Compilation of E' (Stiffness) Values and Thermal Properties of the Films

		Thermal properties			
		First heat run			Second heat run
Film	E' (MPa)	T_g (°C)	T_m (°C)	ΔH_f (J/g)	T_g (°C)
5 wt %	1.4 ± 0.6	−35.55	46.60	17.42	−35.91
10 wt %	3.09 ± 0.7	−34.80	47.43	18.05	−35.42

ΔH_f, heat of fusion.

degraded polymer flakes on the surface of the films. This is because unlike polymers such as poly(lactic-*co*-glycolic acid) (PLGA), which is an amorphous polymer with % WA resulting in bulk degradation, in P(3HO), a semicrystalline polymer, degradation is mulphiphasic, like that observed with the broader PHA family.

In the first stage, spanning a few weeks, the amorphous phase of the polymer began to degrade. This was because the crystalline regions of the polymer were impermeable to water; hydrolysis was, therefore, restricted to the amorphous regions of the polymer and to the fringes of the crystalline region.[26] Next, the crystalline part of the polymer began to degrade; this resulted in the formation of monomers, dimers, and tetramers. Simultaneously, the molecular mass also decreased. Progressively with time, the degradation process developed, and the polymer lost its mass.[27] Such hydrolytic degradation of PHAs have been previously described in the literature and is known to be a slow process when compared to the enzymatic hydrolysis of PHAs.[28] This is because of the relatively high crystallinity of PHAs, which

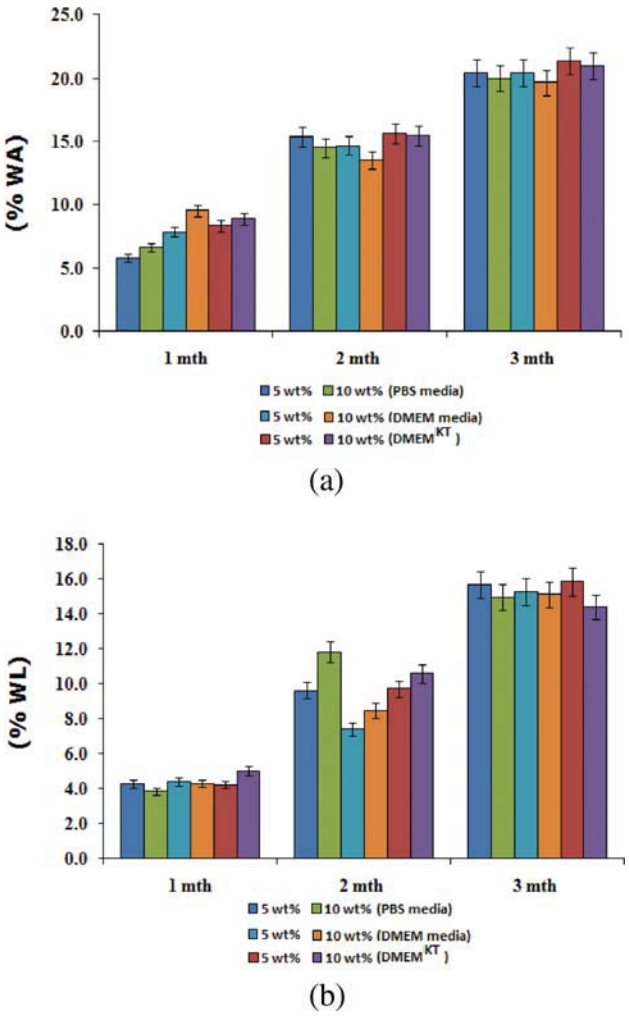


Figure 4 (a) Water absorbed and (b) weight loss by the degrading P(3HO) films during the *in vitro* degradation study in PBS, DMEM, and DMEM^{KT} media. The % WL of the films was monitored for a period of 1, 2, and 4 months. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

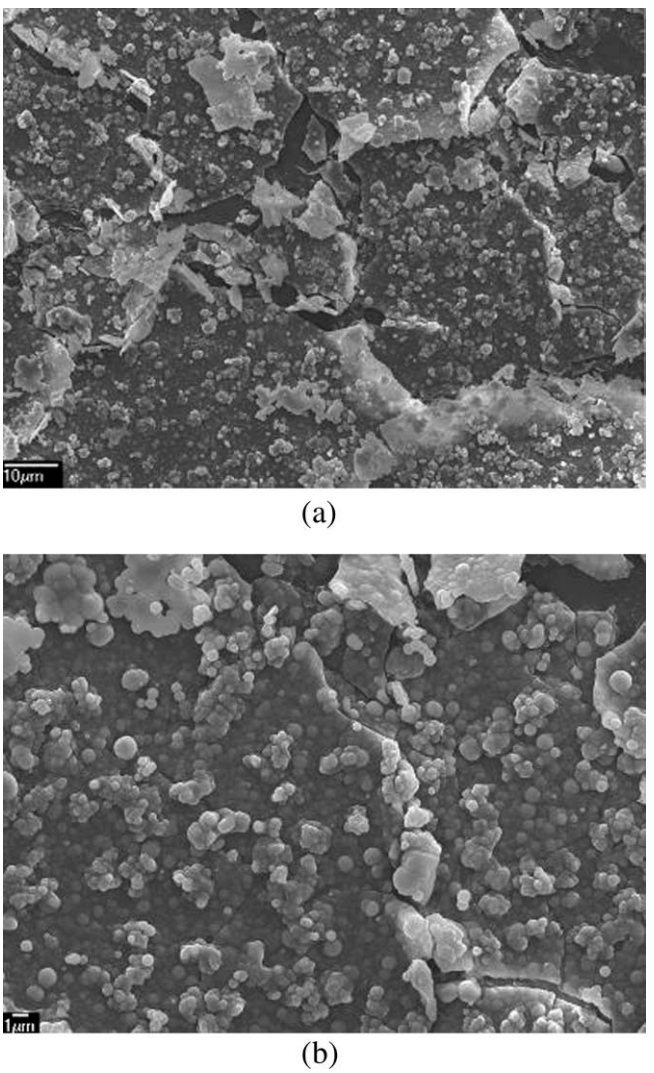


Figure 5 SEM scans at the end of 4 months of incubation for the degrading P(3HO) film (5 wt % in DMEM medium).

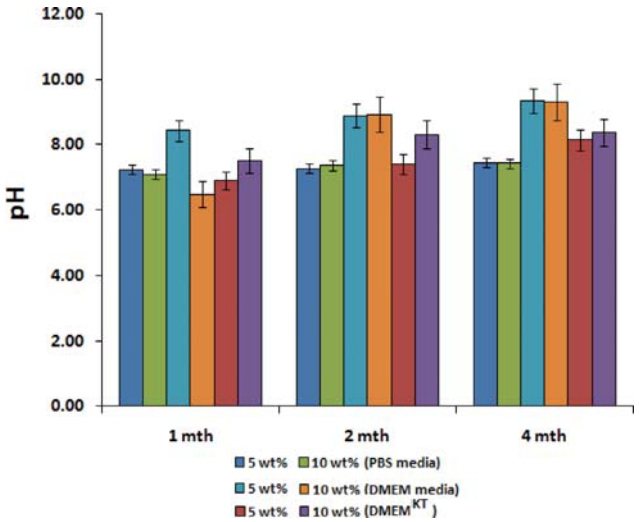


Figure 6 Compilation of the pH of the media (PBS, DMEM, and DMEM^{KT}) in which the films were incubated for the *in vitro* degradation study. The pH was measured over the 4 months of incubation. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

leads to an increased impermeability of water into the crystalline regions,[26] and is also due to the hydrophobic nature of long alkyl pendant chains.[29] This slow hydrolytic degradation could be a reason why the fabricated films lost only 15% of their weight by the end of the degradation studies. However, % WA and % WL 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) and P(3HB-*co*-3HV) samples were incubated for 180 days at 37°C in aqueous media.[30,31] Marios et al.[28] studied the *in vitro* degradation of the P(3HO) film containing 3 mol % 3HHx in water and PBS. The films showed a negligible mass loss of less than 1% after 24 months of incubation.[28] Numerous factors, such as stereoregularity, molecular mass, monomeric composition, and crystallinity of the polymer, affect the biodegradability of PHAs. Studies carried out by Mochizuki and Hirami[32] and Tokiwa and Calabia[33] showed that the biodegradation of PHAs was influenced by the chemical structure (i.e., the presence of functional groups in the polymer chain, hydrophilicity/hydrophobicity balance) and the presence of ordered structures, such as crystallinity, orientation, and morphological properties.[32,33] Usually, the degradation of the polymer decreases with the increase of highly ordered structure, that is, increased crystallinity. Because the P(3HO) used in this study had a lower crystallinity than P(3HB) and P(3HB-*co*-3HV),[30,31] the fabricated P(3HO) films could be expected to be more degradable than P(3HB) and P(3HB-*co*-3HV), as described in literature.

In this study, the pH of all the three media, PBS, DMEM, and DMEM^{KT}, in which the neat films were incubated, increased progressively with time (Fig. 6). The degradation of the P(3HO) films resulted in the production of 3HO or 3-hydroxyoctanoic acid (HA), depending on the pH of the media. HA's dissociation upon release after degradation can be represented as follows:

$$\mathrm{HA(aq)} \leftrightarrows \mathrm{H^{+}aq} + 6A\bullet$$

The pK_a for HA is 4.89. Because the pH of all three media was 7.4, which was greater than the pK_a of the acid (4.89), most of the acid molecules would have existed in the form 3HO, a base, and led 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 E' of the polymer increased after 2 months of incubation and, again, showed a reduction in the modulus after 4 months of incubation, as shown in Figure 7. Although E' decreased at 4 months, the value was still higher than those observed for the undegraded films. This increase in E' of the films after degradation could have been due to the aging of the polymer.[34] The aging in PHAs occurred because of the secondary crystallization of the PHAs, which involved the development of the interlamellar secondary crystals in the amorphous region of the semicrystalline polymer. The small crystallites produced restricted the motion of the polymer chains in the amorphous regions and, thereby, reduced the mobility of the polymer chain

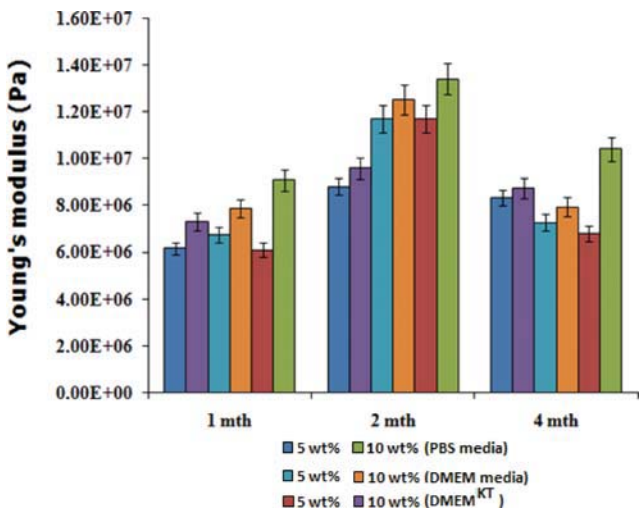


Figure 7 E' of the degraded samples during the *in vitro* degradation study. The samples were thermostatically incubated in (A) DMEM, (B) DMEM^{KT}, and (C) PBS media for a period of 1, 2, and 4 months. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

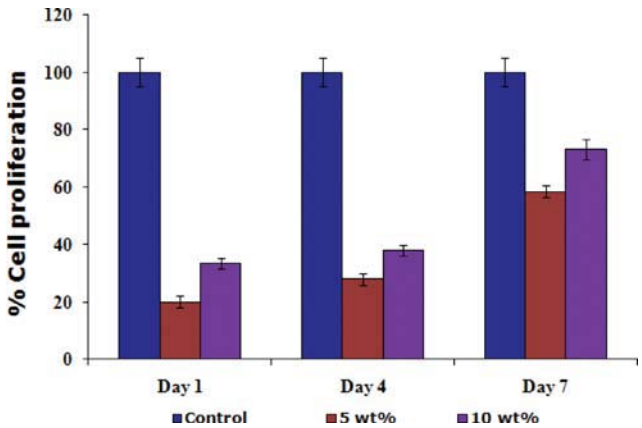


Figure 8 Proliferation study of the seeded HaCaT cells on the fabricated P(3HO) films at days 1, 4, and 7. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

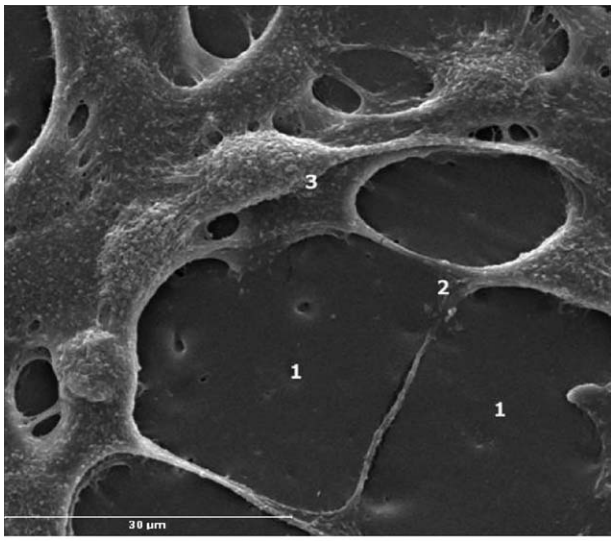
segments. This raised the modulus and increased the brittle nature of the material.[35] Many PHAs have been reported to show aging behavior.[23,35–37] For example, when Asrar et al.[23] aged the copolymer P(3HB-*co*-8.1 mol % 3HHx) for 11 days at room temperature, its E' increased from 18.8 to 22.8 MPa. Alata et al.[37] studied the aging effect on the mechanical properties of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)] containing different molar percentages of 3HHx, ranging from 5 to 18 mol %. After 60 days of aging, they found that the percentage elongation at break had decreased and the tensile strength had increased for all the copolymers. Similar observations were made for P(3HB) and a copolymer of P(3HB-*co*-3HV).[35]

This aging effect exhibited by the films in this study was not desirable. This could be overcome by the incorporation of suitable hydrophilic polymers or plasticizers into the films. Such addition could also be employed to tailor the degradation rate of the films, as the addition of amorphous or hydrophilic additives would lead to higher % WA 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]

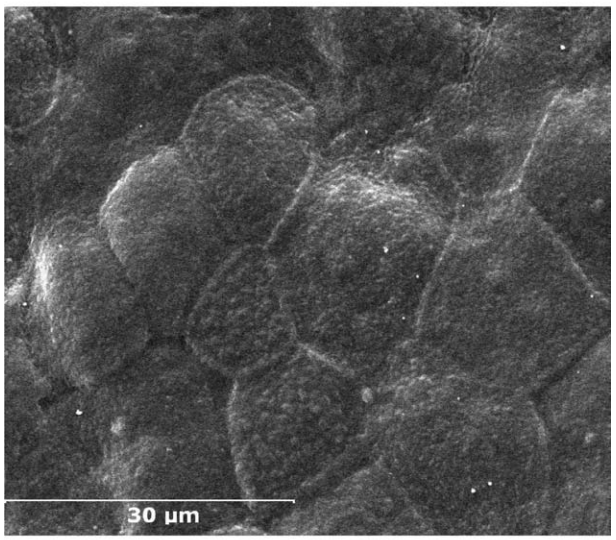
In tissue engineering, it is important that the effect of the degradation products on cellular behavior is considered for a comprehensive biocompatibility evaluation of the implant polymers. Studies were carried out by Sun et al.[39] in 2007 on the cellular responses of the mouse fibroblast cell line L929 to the PHA degradation products, that is, oligohydroxyalkanoates (OHAs). They found that the cytotoxicity of OHAs decreased with increasing OHA side-chain length; this indicated that mcl-OHAs containing PHA, such as P(3HB-*co*-3HHx) and mcl-PHAs, are more biocompatible than scl-PHAs. Therefore, the degradation products of the fabricated neat P(3HO) films in this study could 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 cell and material interfacial relationships, particularly related to protein adsorption. This is because most mammalian cells are anchorage dependent and need



(a)



(b)

Figure 9 SEM images of the seeded HaCaT cells on the fabricated films. (a) Seeded HaCaT cells showing its attachment and proliferation on the film and (b) arrangement of cells in horn sheets: (1) uncovered polymer matrix, (2) cell layer, and (3) spreading of the cell.

TABLE III
Investigated or Potential Biomaterials in Heart Tissue Engineering

Polymers	E or T	E' (or stiffness)	Tensile strength	References
Poly(glycolic acid)	T	7–10 GPa	70 MPa	49 and 50
PLLA or PDLLA	T	1–4 GPa	30–80 MPa	50
P(3HB)	T	2–3 GPa	36 MPa	51
PGS	E	0.04–1.2 MPa	0.20.5 MPa	52
Collagen fiber (tendon/cartilage/ligament/bone)	E	2–46 MPa	1–7 MPa	50 and 52
Collagen gel (calf skin)	E	0.002–0.022 MPa	1–9 kPa	53
Rat myocardium	E	0.001–0.14 MPa	30–70 kPa	54 and 55
Human myocardium	E	0.02–0.5 MPa	3–14 kPa	56 and 57
P(3HO) (5 wt % film)	E	1.4 MPa	1.8 MPa	This study

T, thermoplastic; E, elastomeric. Adapted from ref. 46.

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

The *in vitro* cell biocompatibility studies of the films were carried out by the seeding of HaCaT cells onto the films and the study of their proliferation over durations of 1, 4, and 7 days. The results (Fig. 8) show that the cell proliferation increased progressively on all of the films, and at day 7, the proliferations were 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 (Fig. 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 the films and their suitability for the proposed applications

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 on the basis of their stiffness, that is, their E' values. Table III compares the properties of various biomaterials that have been used for cardiac tissue engineering with the fabricated 5 wt % P(3HO) film in this study. Other biomaterials being studied for this application seem to be either too stiff, such as poly(glycolic acid), poly(L-lactic acid) (PLLA), and poly(DL-lactide) (PDLLA), whose E' values range in the gigapascals or are very soft,

TABLE IV
Potential Biomaterials in Skin Tissue Engineering

Polymer	E' (MPa)	Tensile strength (MPa)	Reference
P(3HB)	1640	12.9	13
P(3HB)/Ha	401	3.3	13
P(3HB)/CH	334	3.3	13
P(3HB)/P(4HB)	214	8.1	13
P(4HB)	632	44	13
P(4HB)/Ha	11.9	3.4 (yield strength = 1.5)	13
P(4HB)CH	29.2	4.1 (yield strength = 2)	13
CH films containing fucoidan	—	7.1	61
P(3HO) (5 wt % film)	1.40	3.3	This study
P(3HO) (10 wt % film)	3.09	Not determined	This study

A dash indicates that the value was not quoted. Adapted from ref. [13].

like collagen gel, whose E' value is in the range 0.002–0.022 MPa. The stiffness of the fabricated P(3HO) films, in particular, the 5 wt % film, was comparable to the higher end stiffness value, from 0.04 to 1.2 MPa, of poly(glycerol sebacate), 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 the grafting of acrylamide and carboxyl ions with plasma treatment[48] and by blending it with other biocompatible elastomeric polymers such as collagen, so its mechanical properties can match either the stiffness of the heart muscle at the beginning of diastole (stiffness = 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) [poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate)], and P(3HB-*co*-3HHx).[13,18] However, until now, P(3HO) has never been studied in the context of skin tissue engineering. To our knowledge, this is the first such study of a homopolymer of P(3HO) and its biocompatibility with HaCaT cells.

Even though 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 in the delivery of 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, the 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 E' value, and the tensile strength of the fabricated P(3HO) films were found to be lower compared to human skin.[60] One possible approach for improving the mechanical properties of the films to match the properties of human skin would be to blend P(3HO) with other biocompatible and mechanically suitable materials for skin, such as chitosan (CH), hyaluronic acid (Ha), and or poly(4-hydroxybutyrate) [P(4HB)].[13] P(4HB)/Ha and P(4HB)/CH blends have been found to have mechanical properties suitable for human skin. Table IV compares the properties of various biomaterials that have been used for skin tissue engineering.

CONCLUSIONS

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 behavior, and biocompatibility properties. Mechanically, the films were found to possess a 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 suggest that P(3HO) films may have potential for soft tissue engineering.

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