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Review

MEDICAL APPLICATIONS OF BIOPOLYESTERS POLYHYDROXYALKANOATES*

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Abstract Microbial polyhydroxyalkanoates (PHAs) are a family of biopolyesters produced by many wild type and engineered bacteria. PHAs have diverse structures accompanied by flexible thermal and mechanical properties. Combined with their *in vitro* biodegradation, cell and tissue compatibility, PHAs have been studied for medical applications, especially medical implants applications, including heart valve tissue engineering, vascular tissue engineering, bone tissue engineering, cartilage tissue engineering, nerve conduit tissue engineering as well as esophagus tissue engineering. Most studies have been conducted in the authors' lab in the past 20+ years. Recently, mechanism on PHA promoted tissue regeneration was revealed to relate to cell responses to PHA biodegradation products and cell-material interactions mediated by microRNA. Very importantly, PHA implants were found not to cause carcinogenesis during long-term implantation. Thus, PHAs should have a bright future in biomedical areas.

Keywords: PHB; PHA; Polyhydroxyalkanoates; Tissue engineering; Biomaterials; Implants.

INTRODUCTION

Microbial polyhydroxyalkanoates (PHAs) are a family of biopolyesters with diverse structures and properties[1]. So far, at least six members of PHAs including poly(3-hydroxybutyrate) (PHB), poly(4-hydroxybutyrate) (P4HB), copolymers of 3-hydroxybutyrate and 3-hydroxyvalerate (PHBV), copolymers of 3-hydroxybutyrate and 4-hydroxybutyrate (P3HB4HB), copolymers of 3-hydroxybutyrate and 3-hydroxyhexanoate (PHBHHx) and terpolymers of 3-hydroxybutyrate, 3-hydroxyvalerate and 3-hydroxyhexanoate (PHBVHHx) have been produced in large scale to be available for application studies.

Over the past 20 years, many researches have been directed to develop medical applications using PHAs. The application of PHAs as *in vivo* implants has ranged from medical devices such as sutures, adhesion barriers, and valves to guided tissue repair, regeneration devices such as cardiovascular patches, articular cartilage repair scaffolds, bone graft substitutes and nerve guides[2, 3]. PHAs are the only kind of polymers produced purely by

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microbial synthesis[4]. The mechanical properties of PHAs can be adjusted by using various monomers and various monomer ratios to meet implant requirement needs. Meanwhile various surface modification technologies including treatments using lipases, acidic or alkali agents, chemical grafting, physical attachment of ligands (and/or receptors) as well as the formation of PHA nanofibers have been developed to improve their tissue regeneration effects. And the advantages of PHAs also include no toxicity of biodegradation products and no risk of carcinogenesis. In 2007, P4HB suture produced by TEPHA company gained its approval from United States Food and Drug Administration (FDA), indicating a promising future for PHA practical application in medical fields[3].

Therefore, the purpose of this review is to summarize all the achievements of PHA implant studies in the last two decades, to provide the mechanism of cell responses to PHA materials, and to predict the future for PHA medical application. Among references cited, many of them are from the authors' own research.

PROPERTIES OF PHA

Thermal and Mechanical Properties

PHAs (general structure shown in Fig. 1) are structurally diverse polyesters produced by many bacteria as reserved carbon and energy sources under unbalanced growth conditions[5]. Depending on the carbon chain length of the monomer compositions, PHAs are classified as short-chain-length (scl) PHAs (3–5 carbon atoms) and medium-chain-length (mcl) PHAs (6–14 carbon atoms)[6]. So far, over 150 monomers of different structures have been reported, including some monomers with functional groups such as unsaturated bonds, benzene, halogens, epoxides and cyclic chemicals[1]. The diverse structures provide PHA family with wide range of melting temperatures (T_m, from 53 °C to 179 °C), glass transition temperatures (T_g, from −51 °C to 4 °C), Young's modulus (from 0.2 GPa to 149 GPa), tensile strength (from 10 MPa to 104 MPa) and elongation at break ranging from 5% to 1080% (Table 1)[7–9]. Generally, the scl-PHAs have thermal and mechanical properties close to conventional plastics polyethylene or polypropylene, while the mcl-PHAs are similar to elastomers and rubbers[5, 6, 10]. One of the benefits for PHA tissue engineering is the flexible properties that can be tailor-made with different combinations of building blocks. Coupled with various fabrication techniques, PHA can be designed as scaffolds for both hard and soft tissues applications.

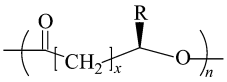

Fig. 1 General molecular structure of PHA[2]
x = 1, 2, 3, yet x = 1 is most common; n can range from 100 to several thousands. R is variable. When x = 1, R = CH_3, the monomer structure is 3-hydroxybutyrate, while x = 1 then R = C_3H_7, it is a 3-hydroxyhexanoate monomer.

Biocompatibility and Biodegradation Behaviors

In 1993, Gogolewski *et al.* reported the tissue response and *in vivo* degradation of PHB and PHBV (5 mol%–22 mol% HV monomer content), compared with polylactides (PLA). The PHA and PLA were implanted subcutaneously in mice. No acute inflammation, abscess formation, or tissue necrosis was observed in tissues adjacent to the implanted materials. The PHB and PHBV were degraded much less (15%–43%) than the PLA did following 6 months of implantation. Weight loss during implantation ranged from 0–50% for PLA, whereas for the PHAs weight loss ranged from 0–1.6%[11]. Copolymer of 3-hydroxybutyrate and 3-hydroxyoctanoate, short as poly(3HB-*co*-3HO), showed minimal tissue reaction after 2 weeks, and no macrophage was observed at the implant sites. Histological analysis revealed that the implants were encapsulated by a thin loose connective tissue layer (four to six fibroblasts cell layers thick). During 40 weeks of implantation, the amount of tissue adherent did not increase. Slow and homogeneous hydrolytic breakdown of the polymer was observed on the surface of the polymer devices[12]. Similar with the other PHAs, there was no evidence of macrophages around the subcutaneous implants PHBHHx during the 6 months *in vivo* period. PHBHHx showed different *in vivo* degradation behaviors from PHB. High hydrolysis rate in amorphous regions in PHBHHx produced more low-

molecular-weight compositions than in PHB, this, therefore, brought the remarkable decrease of M_n in PHBHHx (Table 2)[13]. It is worth mentioning that PHAs exhibited less significant acute inflammatory response than PLA did[13]. The hydroxyl acids released from PHAs were much less acidic and less inflammatory than the lactic acid released from PLA degradation[14, 15].

Table 1. Comparison of various PHA polymer properties[7–9]

Polymer	T_m (°C)	T_g (°C)	Young's modulus (GPa)	Tensile strength (MPa)	Elongation at break (%)
P(3HB) or PHB	179	4	3.5	40	5
P(3HB-*co*-3 mol% 3HV)	170	–	2.9	38	–
P(3HB-*co*-9 mol% 3HV)	162	–	1.9	37	–
P(3HB-*co*-14 mol% 3HV)	150	–	1.5	35	–
P(3HB-*co*-20 mol% 3HV)	145	–1	1.2	32	–
P(3HB-*co*-25 mol% 3HV)	137	–	0.7	30	–
P(3HB-*co*-3 mol% 4HB)	166	–	–	28	45
P(3HB-*co*-10 mol% 4HB)	159	–	–	24	242
P(3HB-*co*-16 mol% 4HB)	–	–	–	26	444
P(3HB-*co*-64 mol% 4HB)	50	–	30	17	591
P(3HB-*co*-90 mol% 4HB)	50	–	100	65	1080
P(4HB) or P4HB	53	–51	149	104	1000
P(3HB-*co*-10 mol%3HHx)	127	0	–	21	400
P(3HB-*co*-17 mol%3HHx)	120	–2	–	20	850
P(3HHx-*co*-3HO)	61	–	–	10	300
P(3HB-*co*-3HV-*co*-3HHx)	113	1.3	0.3	5.1	263
Polypropylene	170	45	1.7	34.5	400
Polyethylene-terepthalate	262	3400	2.2	56	7300
Polystyrene	110	21	3.1	50	–
LDPE	130	–30	0.2	10	620

Table 2. Changes in molecular weights of PHA implants over a six month period[13]

Materials		M_w	M_n	M_w/M_n
PLA	Initiate	174000 ± 3%	47200 ± 3%	3.7
	1month	159000 ± 14%	43300 ± 2%	3.7
	3 month	156000 ± 17%	27100 ± 1.1%	5.7
	6 month	15300 ± 5%	3600 ± 1.3%	4.2
PHB	Initiate	534000 ± 8%	144900 ± 2%	3.6
	1month	457000 ± 0.6%	128000 ± 7.6%	3.6
	3 month	300000 ± 13%	101000 ± 0.9%	2.9
	6 month	216000 ± 9%	81200 ± 5%	2.7
PHBHHx	Initiate	409000 ± 6%	168000 ± 10%	2.4
	1month	368000 ± 4%	118000 ± 10%	3.1
	3 month	236000 ± 3%	56000 ± 20%	4.2
	6 month	178000 ± 5%	20600 ± 8%	8.6

Recently, different modification methods were developed for better performances of PHAs. Various chemical surface modification techniques including alkaline hydrolysis[16], ammonia plasma[17] and lipase treatment[18] have been applied to improve surface hydrophilicity for better cell growth. However, molecular weights of the materials were always reduced by these chemical treatments, which damaged mechanical properties of the polymers[19]. Physiologically a lot of amphiphilic proteins are embedded in phospholipid monolayer surrounding of amorphous PHA cores to regulate polymer synthesis, accumulation and degradation[20–22]. Unspecific binding of PHA granule associated proteins, such as Phasin (PhaP) and PHA

synthesis regulator (PhaR), provides an effective physical modification method. The fusion protein PhaP-Arg-Gly-Asp (RGD) as the binding domain was immobilized on the polymer substrate surface to deliver RGD as the adhesion promoting domain for better growth of the attached cells[23]. The PhaP-RGD coating led to more homogeneous spread of cells, better cell adhesion, proliferation and chondrogenic differentiation of human bone marrow mesenchymal stem cells (hBMSCs) in the scaffolds compared with those of PhaP coated or uncoated scaffolds in serum minus chondrogenic induction medium. Homogeneously distributed chondrocytes-like cells forming cartilage-like matrices were observed on/in the PhaP-RGD coated scaffolds after 3 weeks[24]. Very recently, PhaR fused with Lys-Gln-Ala-Gly-Asp-Val (KQAGDV) oligopeptide was also proven to have a similar effect on polymer cytocompatability improvement with PhaP-RGD[25].

The degradation rate of PHAs can be tailored by blending with rapid degraded materials, such as PLA[26], gelatine[27], *etc*. Chemical grafting using maleic anhydride to form maleated PHBHHx accelerated degradation. The maleated PHBHHx lost 21.4% of its original weight after 21 weeks while PHBHHx just lost 7.3% in simulated body fluid (SBF) at 37 °C[28]. Ultraviolet (UV) radiation could be another method to achieve controlled degradation of PHAs. When UV radiation was applied directly to PHBHHx powder, significant molecular weight (M_w) losses and M_w distribution increase were observed with the powder. After 15 weeks, films prepared from 8 and 16 h UV-treated PHBHHx powders lost 8% and 13% of their original weights, respectively, while the untreated PHBHHx films lost only 1% of its weight. Interestingly, this radiation did not damage the mechanical properties of films made of the UV-radiated powder. In comparison, the PHBHHx films subjected to direct UV radiation became very brittle although their degradation was faster than that of the PHBHHx powders subjected to direct UV radiation. Better growth of fibroblast L929 was observed on films prepared from UV-radiated powders, due to the increasing hydrophilic functional groups generated by increasing polar groups C—O and C—OH[29].

IN VIVO STUDY FOR PHA MEDICAL IMPLANTS

Heart Valve Tissue Engineering

The most common treatment for end-stage valvular diseases is surgical replacement by either mechanical valves or xeno- or homo-grafts. The advantage of mechanical valves is good structural durability and the disadvantage is the risk of side effects, such as prosthetic valve endocarditis, and thromboembolic complications, caused by their non-physiological surfaces and flow abnormalities[30]. All of the succedaneous valves are unable to grow into larger volume with the body, thus child patients with outgrow replacement valves need repeated surgeries[31]. The goal of heart valve tissue engineering is to regenerate a functional growing heart valve grafts[32]. Decellularized extracellular matrix[33, 34], polyglycolic acid (PGA)[35], polylactic acid (PLA)[35], PLGA[36], polycaprolactone (PCL)[37] and PHA[38–41] have been utilized to shape into heart valve and support cell growth and even differentiation. Among PHA family, medium-chain-length PHAs (mcl-PHA) are more flexible materials, which are suitable to function as leaflets inside a tri-leaflet valve. Implantation of a whole trileaflet tissue-engineered heart valve using copolyester of 3-hydroxyhexanoate and 3-hydroxoctanoate [P(3HHx-*co*-3HO)] with seeded autologous cells in the pulmonary position in a lamb model was found to be functional up to 120 days, with no thrombus formation and mild stenosis[38]. Blending with other polymers can improve the properties and performance of PHA heart valve. When PGA was used to blend with P(3HHx-*co*-3HO), non-progressive, valvular regurgitation was observed after 6 months of implantation[42]. In another study, PHB was used to impregnate the decellularized porcine aortic matrix, the hybrid tissue valves were revealed with better biological properties including lower *in vitro* plasmatic coagulation cascades and less calcification[43]. Similarly, decellularized porcine aortic valves coated with PHBHHx were able to maintain their original shapes, they were covered by a confluent layer of cells, and had less calcification than the uncoated control up to 16 weeks in pulmonary position in sheep.

The PHBHHx coating improved tensile strength of the hybrid valve and promoted the repopulation of the recipient cells resembling native valve tissue (Fig. 2)[44]. Most interestingly, some tissue engineered heart valve

exhibited *in vivo* volume expansion with time. It was reported that tri-leaflet heart valves made from P(4HB) coated with PGA non-woven mesh with autologous ovine myofibroblasts and endothelial cells sequentially seeded were grown for 14 days in a pulse duplicator *in vitro* system under gradually increasing flow and pressure conditions[45]. The size of constructs increased from 19 to 23 mm during 20 weeks period of implantation in lamb, indicating the possibility to develop an engineered valve that can grow with the heart valve volume of children[45].

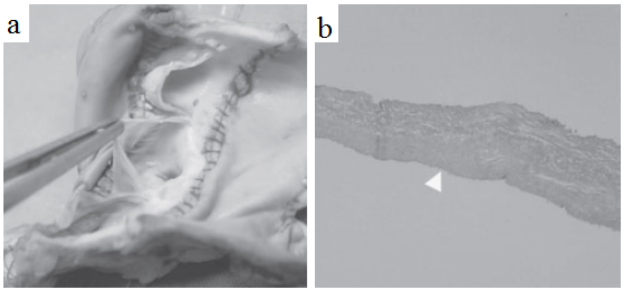

Fig. 2 The decellularized porcine aortic valve/PHBHHx hybrid heart valve[44] (a) the hybrid valve conduit was covered by a confluent layer of cells 16 weeks after implantation in pulmonary position in sheep without cardiopulmonary bypass, (b) PHBHHx coating reduced calcification and promoted the repopulation of the recipient's endothelial cells resembling native valve tissue. The white arrow indicated the cell layer. (Magnification: ×40). (Reproduced with permission from the publisher).

Vascular Tissue Engineering

The pathology that affects the blood vessels is a leading cause of morbidity and one of the major causes of death world-wide[46]. Usually, these cardiovascular disorders are treated through cardiac and peripheral bypass surgery, by which the vascular failure are replaced by autologous or xenogenous veins, arteries, or artificial prostheses. However, the autologous vessels are very limited, while the xenogenous ones may cause immunological rejection and finally lead to graft failure. Synthetic vascular grafts such as polytetrafluoroethylene (ePTFE) or Dacron have been used successfully as a substitute for large arteries (> 6 mm internal diameter)[47]. But when smaller diameter vessels are used, they often cause high thromobogenicity and thus reduced their patency after implantation[48]. The smaller diameter grafts (< 6 mm) have to confront more challenges, such as the problem of incomplete endothelialization and rapid closure owing to low blood flow conditions[49]. UV radiation and surface coating have been applied to solve these problems[50]. However, there is still a long way to go to achieve successful small diameter blood vessel replacement.

Generally, nature arteries are composed of three layers, the intima, media and adventitia. The intima forms the layer closest to the blood flow and consists of a lining of endothelial cells to prevent blood coagulation, while the media contains a dense population of smooth muscle cells that organized concentrically to maintain the contractibility of the vascular. Adventitia, which contains extracellular matrix together with fibroblasts, small blood vessels and nerves, functions to maintain the shape and nutrition transport. Thus, a functional vascular substitute mimicking the nature artery should contain an anti-coagulated intima with flexible media and a porous adventitia for loose connective tissue to grow in. The emergence of tissue engineering opens new possibilities in reconstructive vascular grafting and offers an alternative resource to meet these requirements[51]. Based on careful selection of biodegradable materials and proper processing procedure, scaffolds with appropriate structure and mechanical properties could be fabricated, with seeded cells together to accomplish the function of damaged artery[52–54].

Early in the study of PHA vascular tissue engineering, flexible mcl-PHAs including poly-3-hydroxyoctanoate (PHO) and P4HB were used[55]. Shum-Tim *et al.* modified PGA porous scaffolds by coating with PHO which were used to fabricate 7 mm diameter PHO/PGA tubular scaffolds. The autologous cell-polymer vascular constructs were used to replace 3–4 cm abdominal aortic segments in lambs. After 101 days

in vivo, all of the grafts remained potent, and no aneurysm was observed. The mechanical strain-stress curve as well as the DNA and collagen amounts of the PHO/PGA aorta approached that of the native vessel. Elastic fibers along the vessels in the medial layer and endothelial specific von Willebrand factor on the luminal surface were observed in the histological straining photos[55].

One of the most exciting events in pure PHA vascular tissue engineering was reported by Stock *et al*. in 2000[56]. In their experiments, autologous vascular cells isolated from ovine peripheral veins were seeded on porous P4HB patches and the patches were implanted into the proximal pulmonary artery in a sheep model. After 169 days, a near-complete resorption of the polymer with formation of organized and functional tissues was demonstrated without thrombus, stenosis or dilation[56]. Opitz *et al.* went a step further in 2004, by seeding vascular smooth muscle cells (vSMCs) onto P4HB scaffolds and incubated them in a pulsatile flow bioreactor[57]. They observed confluent layered tissue formation accompanied by improved mechanical properties which approached those of native aorta[57]. In 2007, Mendelson *et al.* achieved a successful patch repair[58]. They seeded autologous ovine blood-derived endothelial progenitor cells (EPCs) and bone marrow-derived mesenchymal stem cells (MSCs) onto P4HB coated PGA nonwoven biodegradable mesh scaffolds and cultured for 5 days in a laminar fluid flow system to construct P4HB/PGA tissue engineered vascular patches[58].

Then these patches were implanted into the wall of sheep pulmonary artery for 6 weeks. Surface thrombus formation and macrophage infiltration happened in the first week. Early angiogenesis (microvessel formation) began in the second week *in vivo*. After 4 weeks, the implanted patches were filled with glycosaminoglycans and collagen, and their luminal surfaces were covered by host artery-derived pannus containing alpha-SMA-positive cells and laminated elastin. During the tissue repair period, polymer scaffold degradation was almost complete[58].

PHBHHx has shown improved hemocompatibility and cytocompatibility compared with similar materials[59]. Better growth of human umbilical vein endothelial cells (HUVECs) and smooth muscle cells from rabbit aorta (RaSMCs) were promoted by surface modification *via* fibronectin coating and/or ammonia plasma treatment. HUVECs formed a confluent monolayer on modified PHBHHx film, indicating the promises of PHBHHx based material for luminal surface and vascular grafting[17]. More excitingly, it was demonstrated that 20%-HHx containing PHBHHx not only supported RaSMCs proliferation, but also induced its change to contractile phenotype[59]. The intravascular biocompatibility of PHBHHx was evaluated by Wu *et al.* in 2008. In their experiment, decellularized xenogenic vascular scaffolds were coated with PHBHHx and implanted in the rabbit abdominal aorta for 12 weeks (Fig. 3). The hybrid patches maintained original shapes, covered by confluent layer of cells, with less calcification than uncoated control ones[60]. Further investigation of vascular replacement in animal model is being carried out in the authors' lab. PHBHHx tubes with two/three layers for use under different microenvironment clues are fabricated. BMSCs seeded on the inner surface could differentiate towards vascular smooth muscle cells and orientate along the direction of the align fibers, which simulated the media of natural arteries. And the outer layer could form the loose connective tissue as the adventitia of nature arteries (unpublished results).

Bone Tissue Engineering

The principal role of the skeleton is to provide structural support for the body. Hollow tube bones provide great strength and durability against axial compression forces while at the same time minimize weight. The ultimate tensile strength of bone approaches that of cast iron, and its capacity to absorb and release energy is twice that of oak, yet the weight of bone is only one third that of steel[61, 62]. Three distinctly different cell types can be found within bones: the matrix-producing osteoblast, the tissue-resorbing osteoclast, and the osteocyte, which accounts for 90% of all cells in the adult skeleton[61]. Bone tissues have excellent self-regeneration ability. However, when bone defects exceed a critical size, impaired bone formation can occur and surgical intervention becomes mandatory[63]. Bone tissue engineering provides the most promising alternative to conventional bone transplants, avoiding the risks of disease transfer and donor site morbidity[63].

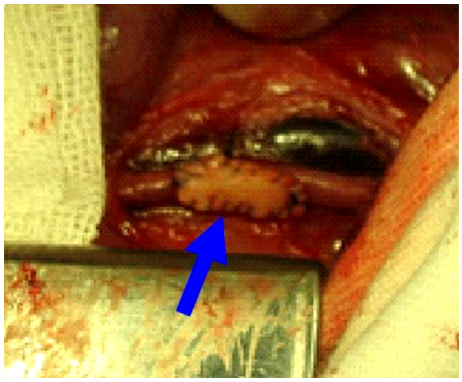

Fig. 3 The PHBHHx/decellularized vascular hybrid patch implanted in the rabbit abdominal aorta[60] (Reproduced with permission from the publisher)

Short-chain-length PHAs (scl-PHA) such as PHB and PHBV with relatively high mechanical strength were first applied for bone scaffold fabrication. Rapid proliferation of osteoblasts was observed on PHB material[64]. In 1991, Doyle *et al*. implanted PHB into rabbits to demonstrate that PHB could produce a consistent favorable bone tissue adaptation response with no evidence of an undesirable chronic inflammatory response after implantation periods up to 12 months[65]. The implant surface was covered with highly organized new bones[66]. Similarly, in another study of PHB implantation in anterior skull base of a pig，fine hydroxyapatite crystallites were found to form at the interface[67]. It was reported in 2003 that the proliferation and mineralization of osteoblasts were observed on PHBV foams with large pore sizes (300–500 μm) rather than on the one with small pore sizes (75–300 μm)[68]. And bone marrow stromal cells (BMSCs) exhibited osteogenic differentiation features including increasing alkaline phosphatase activity and osteocalcin secretion[68]. In their subsequent *in vivo* experiments, porous PHBV scaffolds seeded with rat BMSCs were implanted into defects created in rat femurs, bone regeneration was clearly observed in 6 weeks. Compared with calcium phosphate-loaded collagen (CaP-Gelfix) foams, PHBV inflammation was minimal at all stages and better healing and less fibrous tissue formation were observed throughout the PHBV foam[69].

To further improve the biocompatible and mechanical properties of the scaffolds, hydroxyl apatite (HA) which contributes 65%–70% to the bone matrix, was used as an additional blending material[70–74]. P(3HB-*co*-8%3HV)/HA (30%, *W*/*W*) showed a mechanical compression strength of 62 MPa, which is about the same order of magnitude of several human bones[75]. The most outstanding parameters of growth and differentiation of murine marrow osteoblasts were found on PHB/HA blends containing 10% and 20% HA[76]. Nano-sized HA reinforcing PHBV had even lower inflammatory response and higher levels of mineralization[77]. When PHBV/HA scaffolds were implanted in rabbit tibias, osteoblasts and osteocytes were identified throughout the interface region. Lamellar bone was formed at the interface accompanied by polymer matrix degradation[78]. The thickness of the newly formed bone significantly increased over the period of the experiment from 130 μm at 1 month to 770 μm after 6 months[78]. Besides HA, many other materials were blended with PHB or PHBV, such as polyglactin[79], tricalcium phosphate (TCP)[80, 81], sol-gel-bioactive glass (SGBG)[82], natural coral (NC)[83] and wollastonite[84, 85].

Some other PHAs were also reported to have suitable compatibility with bone related cells. Better attachment, proliferation and differentiation of osteoblasts were observed on PHBHHx compared with PHB[86, 87]. P(3HB-4HB-3HHx) supported even more enhanced osteogenic differentiation of MSCs than PHBHHx did (Fig. 4)[88]. Functional evaluation for bone replacement needs to be conducted to further prove their potential application as tissue engineered PHA bones.

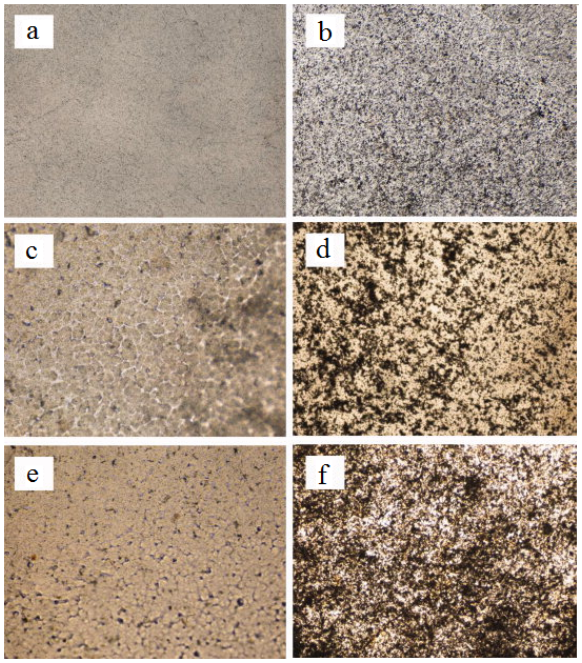

Fig. 4 Von Kossa staining for detection of osteogenic differentiation of human MSCs[88]
Cells were cultured for 3 weeks in regular growth medium as negative controls (a, c, e), or in osteogenic differentiation medium (b, d, f). (a, b) TCPs, (c, d) PHBHHx and (e, f) P(3HB-4HB-3HHx). (Magnification: × 100). (Reproduced with permission from the publisher).

Cartilage Tissue Engineering

Articular cartilage tissue covers the ends of bones in diarthrodial joints for distribution of applied loads. The predominant feature of cartilage is its low cell density with chondrocytes as the exclusive cell type. There is no blood supply, lymphatic system or nerve network in the cartilage tissue. Therefore, once damaged, it has very limited capacity to heal itself, which will often lead to osteoarthritis and functional loss of the joints[89, 90]. Similar to bone tissue engineering scaffolds, an ideal tissue engineered cartilage scaffold should prossess three characteristics: the first one is the high mechanical strength to support temporary functional substitution; the second is the suitable degradation rate to adapt tissue regeneration; and the third is biological activity to induce cell differentiation into chondrocytes.

In vitro study showed that chondrocytes proliferated and preserved their phenotype on PHB, PHBV and PHBHHx[91–93]. They proliferated better on the PHBHHx/PHB scaffolds than on PHB ones[91]. Scaffolds made of PHBHHx/PHB consisting of 60 wt% PHBHHx showed strong growth and proliferation of chondrocytes on the blending materials[94]. PHBHHx had a positive effect on ECM production of chondrocytes in the composite PHB/PHBHHx scaffolds. Second-harmonic generation (SHG) imaging technique combined with confocal fluorescence microscopy (CFM) revealed that PHBHHx in PHB scaffold provided better surface properties for anchoring type II collagen filaments and their penetration into internal layers of the scaffolds[95]. Besides this, crystallinity of the blended polyesters remarkably influenced phenotype maintenance of chondrocytes. The amount of extracellular collagen X, which is the marker of endochondral ossification, decreased with increasing polarity contributed by increasing PHBHHx content in the blend[96]. 3D PHBHHx scaffold coated with a fusion protein PHA granule binding protein PhaP fused with RGD peptide (PhaP-RGD) had more homogeneous spread of cells, better cell adhesion, proliferation and chondrogenic differentiation compared with those of PhaP coated or uncoated scaffolds[97]. Recently, mechanostimulation of human mesenchymal stem cells grown in PHBHHx/collagen hybrid scaffolds was found helpful for tendon tissue regeneration[98]. PHBHHx/collagen scaffolds have also been successfully used to culture hMSC and over an extended period supporting the potential

of this scaffold combination in future tissue engineering applications[99]. The unique surface properties of PHBHHx could even induce chondrogenesis of BMSCs[100].

In 2005, PHBV cartilage scaffolds were evaluated *in vivo* by implantation into full thickness cartilage defects (4.5 mm in diameter and 4 mm in depth). Compared with collagen containing calcium phosphate (CaP-Gelfix), PHBV matrices presented early cartilage formation resembling normal articular cartilage with minimal foreign body reaction[101]. In 2008, PHBHHx was fabricated into 3D scaffolds to evaluate its tissue repair effects in rabbit knee articular cartilage defect model. During 16 weeks implant *in vivo*, the defects filled with PHBHHx scaffolds were observed with white cartilaginous tissue formation. Better surface integrality and more accumulation of ECM including type II collagen and sulfated glycosaminoglycan (sGAG) were achieved in the engineered cartilage constructs with chondrocytes pre-seeded in the PHBHHx scaffolds (Fig. 5)[102]. The greatest challenge of PHA cartilage tissue engineering is still the mechanical property mimicking. It is expected that the new processing methods of materials combined with other modification approaches will enhance the anisotropic elasticity of PHA scaffolds.

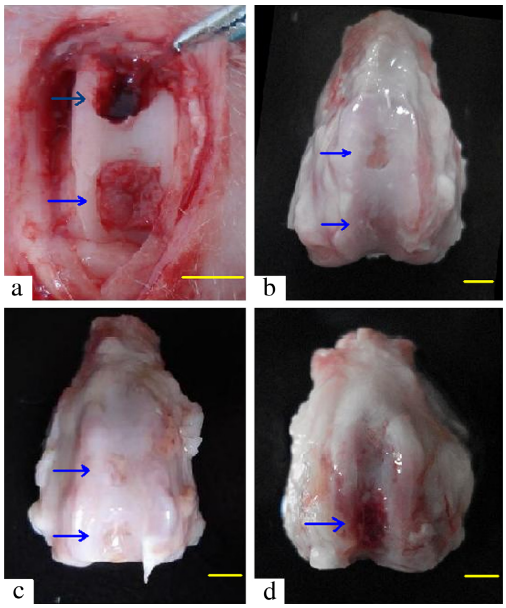

Fig. 5 Macroscopic appearance of full thickness cartilage defects and implant repairs[102] (a) defects created surgically on the femoropatellar groove of the knee joints and filled with a engineered cartilage construct (PHBHHx scaffold with chondrocyte pre-seeded for 10 days) in the lower defect site; (b–d) 16 weeks after operation: (b) two defects with bare PHBHHx scaffolds repair, (c) two defects with engineered cartilage constructs repair, (d) one defect without treatment (Reproduced with permission the publisher) The defects were pointed out with arrows, scale bar: 4 mm.

Besides hyaline cartilage repair, PHA scaffolds had also been applied for fibrocartilage repair. Zhou *et al.* evaluated PHBHHx as tarsal substitute in rats by comparison with commercial acellular dermal matrices (ADM). Both PHBHHx and ADM showed satisfactory repair results in 8 weeks, though the implanted PHBHHx scaffolds caused slight inflammation in the first 2 weeks[103].

Nerve Conduit Tissue Engineering

The peripheral nervous system (PNS) has a much greater capacity than the central nervous system (CNS) for natural regeneration after injury. Clinically, nerve graft, autograft or allograft, is used to bridge the gap in the damaged nerve to enhance the regenerative process. However, this treatment has several drawbacks, including donor site morbidity, scarcity of donor tissue, frequent incidence of donor site neuroma formation, and heavy dependence on immunosuppressants[104]. To overcome the shortages of never grafts, nerve conduit is designed to

aid the regeneration of peripheral nerve tissue. A cylindrical nerve conduit can serve as a connecting bridge for the damaged nerve ends as well as a protective shelter for the regenerating nerve. To date, the marketed nerve conduit products include Neurotube (Synovis), Neurolac (Ascension) and NeuraGen (Integra)[105]. The ideal biodegradable nerve conduit should have the following properties including: maintaining tissue structure integrity, permitting cell communication and subsequent tissue ingrowth during the regenerative processes[106].

In 1999, Hazari *et al.* successfully utilized PHB conduits to repair a 1 cm gap in the rat sciatic nerves[107]. Long nerve gaps (up to 4 cm) in rabbit common peroneal nerves were able to be bridged by PHB conduits with the area of immunostained regenerating fibres bigger than that in the nerve autograft group[108]. To further improve the performance of PHB nerve conduit, Schwann cells were seeded in the inner surface of conduits. The neuron supporting Schwann cells showed strong influences on the nerve regeneration process. The composite conduits seemed to provide an optimal environment for nerve regeneration in the damaged area[109]. Novikova *et al*. in 2008 produced a tubular PHB scaffold with predominantly unidirectional fiber orientation supplemented with cultured adult Schwann cells to aid axonal regeneration after cervical spinal cord injury in adult rats. Although rubrospinal fibers did not enter the PHB, numerous raphaespinal and calcitonin gene-related peptide (CGRP)-positive axons were found within the conduit. The number of fibers crossing the host-graft interface was much more after cells modification[110]. Some extracellular matrix (ECM) protein molecules, such as laminin, fibronectin and collagen, which are proven to be able to enhance adhesion, proliferation and secretion activity of Schwann cells, could also be used to modify PHB conduits[111–113]. PHB tubes filled with glial growth factor (GGF) and suspended in alginate hydrogel supported peripheral nerve regeneration up to 63 days and helped bridge the gaps of 2–4 cm in rabbit common peroneal nerve with GGF released[114]. Especially for long-term regeneration evaluation, GGF addition significantly increased the quantity of Schwann cells, it also promoted axonal regeneration, and improved target muscle reinnervation[115]. Besides PHB, PHBHHx and P3HB4HB have also been found to have good compatibility with neurons and neural stem cells (NSCs)[116]. PHBHHx nerve conduit was evaluated in 1-cm defects in the sciatic nerve of rats by Bian *et al.* in 2009. They designed a two-layer porous PHBHHx conduit with internal wall compact enough to prevent the connective tissues from ingrowth penetration (Fig. 6). This structure also improved the mechanical strength of the conduit[117].

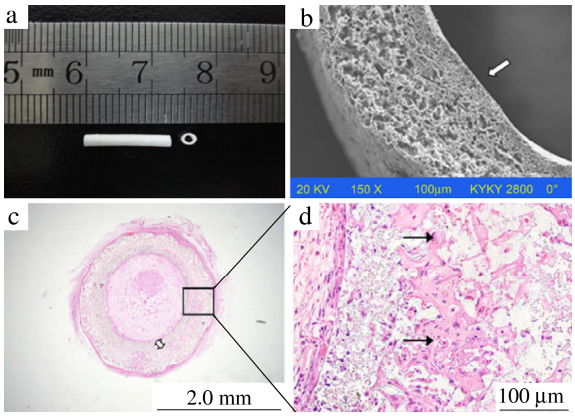

Fig. 6 The PHBHHx nerve conduits bridging 10 mm defects in the sciatic nerve of rats[117] (a) the conduit had a length of 13 mm and internal diameter of 1.5 mm, (b) the conduit had non-uniform wall porosity with outer pore size around 30–50 μm and inner pore size less than 10 μm; the white arrow indicated the internal side, (c, d) HE staining of regenerated nerve fiber in PHBHHx nerve conduit after three months (Reproduced with permission from the publisher)
Black arrows indicated connective tissues grown into the wall of conduit.

For nerve conduit tissue engineering materials, biodegradability is an indispensable factor. The degradation rates should match the rate of new nerve regeneration to avoid disturbing nerve conduction. At present, the degradation of PHB and PHBHHx nerve conduits were shown always slower than the nerve regeneration[108, 117, 118]. Thus, long-term research should be conducted to develop PHA materials that promote tissue regeneration accompanied by an appropriate degradation rate based on tissue regeneration speed. As we mentioned above, UV radiation could be an efficient method to speed up degradation of PHA without damaging their mechanical properties[29].

Esophagus Tissue Engineering

The esophagus is a muscular tube through which food passes from the pharynx to the stomach. It has no digestive function. Esophagus replacement using the present surgical techniques is associated with significant morbidity. Therefore, tissue engineering of the esophagus may provide the solution for esophageal loss. So far, the reported esophagus tissue engineering scaffold materials have included polyglycolic acid (PGA), acellular porcine aorta, small intestine submucosa and hybrids of silicone/collagen[119].

PHAs have shown some muscle inductive regeneration ability. When Mack *et al.* implanted PHB material into rat musculus latissimus dorsi to study ectopic bone regeneration, they accidentally found an increase of slow myosin isoforms, insulin-like growth factor-1 (IGF1) and vascular endothelial growth factor (VEGF), as well as a decrease of muscle inhibitory myostatin (GDF8) in the surrounding muscle tissues[120, 121]. Recently, Ricotti *et al.* studied the proliferation and skeletal myotube formation capability of C2C12 and H9c2 cells on isotropic and anisotropic electrospun nanofibrous PHB scaffold, they found that the aligned nanofibrous mesh decreased the cell proliferation and provided a higher differentiative stimulus[122]. Therefore, PHAs are promising to promote muscular regeneration as esophagus substitutes.

PHBHHx tubes were once implanted in dogs to replace 10 cm esophagus. In a two-month postoperative period, no obvious rejection reaction was observed and cells migrated onto the PHBHHx tubes to form a thick cell layer (Fig. 7)[2]. However, dogs died eventually because the esophagus was gradually blocked up. In the future, degradation rate acceleration of PHA and tissue shaping control during regeneration will be the main challenges.

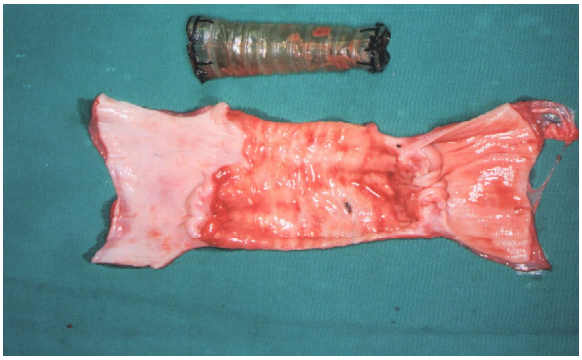

Fig. 7 A thick cell layer formed on the 10 cm PHBHHx esophagus in a two-month postoperative period[2] (Reproduced with permission from the publisher)

MECHANISM STUDY OF PHA PROMOTED TISSUE REGENERATION

Cell Responses to PHA Biodegradation Products

Since PHAs are generally hydrolytic breakdown *in vivo*, the degradation products of PHAs including oligomers (OHAs) and monomers (HAs) must have some influences on the physiological environment of body tissues, and they should be able to affect cell growth states. Generally the oligomers products of PHAs were insoluble. Their cytotoxicity could be evaluated by encapsulating them in liposomes and transferred into cell cytosol. Studies showed that some OHAs, including oligo(3-hydroxybutyrate) (OHB), oligo(3-hydroxybutyrate-*co*-4-

hydroxybutyrate) (O3HB4HB), oligo (3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (OHBHHx) and medium-chain-length oligo (3-hydroxyalkanoates) (OmclHAs), in concentration lower than 20 mg/L did not significantly affect cell viability of murine fibroblast L929, while OHAs over 40 mg/L reduced cell viability with more apoptosis, more cell death, delayed cell cycle and reduced cell proliferation, meanwhile, the cytotoxicity of OHAs decreased with increasing OHAs side chain length[123]. In fact, the amounts of OHAs produced by implanted PHAs always maintained at a concentration lower than 20 mg/L; and they rarely entered the cytosol due to their insolubility. Therefore, cytotoxicity of OHAs could be negligible.

The main cellular influences are contributed to soluble monomers. 3-hydroxybutyrate (3HB) is the most common monomers degraded from PHAs. 3HB is also one of the ketone bodies in the blood. The concentration of 3HB in the blood of an healthy adult is 3–10 mg/100mL[124]. 3HB has been demonstrated as an alternate source of energy in brains when glucose supply is depleted[125]. The dimer and trimer of 3HB in solution were converted completely to monomers when incubated with rat serum or liver homogenate for 10 minutes[126]. Human liver homogenate but not serum could also hydrolyze 3HB dimer and trimer in solution[126]. 3HB could be administered orally or intravenously, as a ketone supplement. An enhanced blood ketone level is beneficial for metabolic disease control including reduction of protein catabolism, appetite suppression, parenteral nutrition, promotion on cardiac efficiency, treatment of diabetes and insulin resistant states, as well as treatment on neurodegenerative disorders and epilepsy. By *in vitro* incubation experiments, 3HB was proven to promote proliferation of various types of cells, including fibroblasts[127], glial cells[128], endothelial cells[127], chondrocytes[127] and osteoblasts[129]. 3HB prevented apoptotic and necrotic cell death of cells in high-density cultures[127]. It also promoted cell proliferation through a stimulatory effect on cell cycle progression that was mediated by a signaling pathway dependent upon increase in $[Ca^{2+}]$[127]. The effect of 3HB on cytosolic calcium concentration could be reduced by nitredipine, an L-type voltage-dependent calcium channel antagonist[128]. The increased influx of extrecellular calcium caused by 3HB also stimulated differentiation and mineralization of osteoblasts[127,129]. Oral administration of 3HB increased alkaline phosphatase (ALP) activity and calcium deposition in serum, decreased serum osteocalcin (OCN), and prevented reduction on bone mineral density (BMD) resulting from ovariectomy in female Wistar rats[129]. An extra protective mechanism was demonstrated that 3HB supported the mitochondrial respiration system by reversing the inhibition of complex I or II[130]. 3HB showed a protective effect against the toxicity of 1-methyl-4-phenylpyridine (MPP) and β-amyloid1-42 in cultured neurons[131, 132].

Another monomer 4HB (also called gamma-hydroxybutyrate), which is the monomer composition of P4HB, has also been found to be widely distributed in brain, heart, lung, liver, kidney and muscle tissues[133]. It has been used for almost 50 years as an intravenous agent with daily dose averages 20–40 g for induction of anaesthesia and for long-term sedation[134]. Therefore, low concentration of 4HB will not be harmful to the body.

Cell-material Interactions

With the development of regenerative medicine, it has become a common sense that biomaterials can not only serve as spatial support substrates to bear the biological cells, but also have a profound impact on cellular behaviors such as cell shape, adhesion, motility, proliferation and differentiation[135]. Study on cell-matrix interaction mechanism has becoming one of the focuses in the field of biomaterial research in recent years[136–138]. Recent studies have shown that biological cells are sensitive to their surrounding microenvironments, they respond to various cues provided by substrate surface properties such as stiffness[139, 140], elasticity[141], anisotropy[142, 143] and topography[144–146], in combination to binding chemical factors including growth factors and ligands[147]. Cell growth supporting substrates including PHAs can be considered as extracellular matrix (ECM) which has important influences on cell behaviors[148].

Among various PHAs, PHBHHx has been found to support better proliferation and ECM production of chondrocytes[99–102] and even induce chondrogenesis of bone marrow-derived MSCs[102]. Unique surface properties[96] and reduced crystallinity were proven to contribute to phenotype maintenance of chondrocytes. Yan *et al.* recently found that MSCs cultured on PHBHHx films for 24 h showed up-regulated expression of

chondrogenic marker genes including *aggrecan, col2*, *sox9*, *col10* and *pthrp* and a similar expression microRNA profiling with chondrocytes[100]. For the first time, they illustrated the cell-substrate interface interactions *via* microRNAs network regulation. MicroRNAs (miRNAs), a class of non-coding RNAs with lengths ranging from 18 to 23 nucleotides, are identified to target mRNA through 5'-seed sequences interacting with microRNAs regulatory elements (MREs) mostly located in the 3' untranslated region of the target mRNA (3' UTR). They play viral and precisely regulating roles in cell proliferation, differentiation, apoptosis and other metabolic processes[149].

However, it is still hard to tell how PHBHHx determines chondrogenesis of MSCs. Some studies showed that the cell shape change was not only the consequence of differentiation, but also played a dominant role in regulating the switch of lineage commitment of hMSC, *via* actin cytoskeleton, RhoA-ROCK signaling pathway, and cytoskeletal tension mechanisms, specifically promoting osteoblastic induction[150–155]. In fact, if the topography of PHBHHx substrate was changed, the differentiation potentials of attached MSCs also changed accordingly. MSCs on the aligned fibers fabricated by electrospinning exhibited much more elongated shape along the aligned fibers, and had distinct cytoskeleton regulation signaling and down-regulated PPAR-gamma signaling for initiating osteogenic stwich[156]. Similar phenomena were observed on microgrooved PHBHHx fabricated using biological microelectromechanical systems (BioMEMS) technology. PHBHHx substrates with 10 μm wide grooves/ridges and 10 μm depths improved osteogenic differentiation of MSCs[157]. However the induction effectiveness of substrate topography alone was very mild and temporary. Substrate surface environment was dynamically changing due to the proliferating cells and depositing ECM. If chemical factors were used in the culture medium, the induction effects of aligned topography were concealed[156].

Another interesting question is how morphologically the cells respond to the surface pattern. More and more evidences have shown that cells sense the substrate by integrin clustering and focal adhesions formation[158]. Integrins are heterodimeric transmembrane receptors composed of eighteen α and eight β subunits that can be non-covalently assembled into 24 combinations connecting an extracellular environment with cytoskeletons. Integrins can activate several signaling pathways independently, but more frequently they act synergistically with other growth factor receptors (GFRs) including insulin like growth factor (IGF), vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF-β), platelet-derived growth factor beta (PDGF-β) and epidermal growth factor (EGF)[159]. Transmembrane integrins become cross-linked in response to mechanical forces of an adhesion. And focal adhesions emerge as diverse protein complexes that provide a dynamically link of the intracellular actin cytoskeletons to an extracellular matrix (ECM)[160]. Further investigation will give more details on integrins expression and activation on the surface of PHAs to depict the specific cell responses.

CARCINOGENESIS ISSUES

Rapid cell growth on PHAs has been observed in most *in vitro* cell cultures and *in vivo* implantation researches, as we summarized above. Will rapid cell proliferation on PHA matrices induce any transformation to cancer cells? This is a crucial question regarding PHA medical application. Peng *et al.* in 2011 investigated the possible carcinogenesis in primary osteoblasts grown on major available PHAs, including PHB, PHBV, PHBHHx, P3HB4HB and PHBVHHx using osteosarcoma UMR-108 cells as a positive control[161]. Multiple tests including mitotic activity analysis by quantitative measurement of BrdU, cancer related genes Ki67, p53 and c-Fos expression analysis, DNA aneuploid formation analysis, telomerase activity and telomere length analysis, were performed to give an integrated assessment. The results showed that no significant difference of cell cycle between the passage 3 to 8 osteoblasts on PHAs. Tumor suppressor genes, Ki67, p53 and c-Fos, which usually are inactive during carcinogenesis, maintained their wild type status. Cells grown on PHA substrates showed some minor cellular nuclear shape change but did not induce DNA aneuploid damage. And after up to 8 times passages, the activity of telomerase was still negative and telomeres turned rather short as normal cells[161]. Therefore, it can be concluded that at least the five tested PHA family members are safe to support cell growth with low risk of cancer cell transformation.

PERSPECTIVES

Great efforts have been made these years to promote PHAs as medical implant materials. It becomes more and more convincing that PHA family members have many advantages as an implant material, such as adjustable physical and thermal properties, long-term maintenance of device shapes, bioactivity for supporting cell growth without risk of carcinogenesis. Because PHAs are produced purely by microorganisms, it will be possible to use metabolic engineering methods to obtain low-cost PHA products and create new family member with new properties.

With the development of biomaterials, the conception of biocompatibility has enriched to become a complex process not just for survivals of cells on/in the material. The performance of tissue-material interaction during the tissue regeneration period should be all taken into account. The tissue regeneration promotion mechanisms of PHA materials have drawn more and more attention recently. At the current stage, it can be concluded that both the degradation products and the material surface topography have contributed to cell behaviors on PHA. It is amazing to see that PHA degradation product 3HB helps to increase cytosolic calcium concentration and protect mitochondrion function, this discovery allows 3HB to be a promising drug candidate for treating bone and nerve system diseases. However, the degradation rate of PHAs is rather slow. Therefore, the effects of 3HB would not be dominant at the early state of cell attachment. Some studies revealed that the cell-material interactions could be responsible for early cell responses. Unique PHA with specific surface characteristics induces specific cellular responses. For instance, the planar PHBHHx films showed some chondrogenic induction effects, while the grooved PHBHHx films would be osteogenic. The signaling transduction processes during cell responses to different surface characteristics will be a major factor for cell responses to materials. Surface engineering approaches will be a significant and powerful tool to design bioactive material for various tissue substitutes.

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