Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate)-based scaffolds for tissue engineering

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Abstract

Development and selection of an ideal scaffold is of importance for tissue engineering. Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx) is a biocompatible bioresorbable copolymer that belongs to the polyhydroxyalkanoate family. Because of its good biocompatibility, PHBHHx has been widely used as a cell scaffold for tissue engineering. This review focuses on the utilization of PHBHHx-based scaffolds in tissue engineering. Advances in the preparation, modification, and application of PHBHHx scaffolds are discussed.

Key words: PHBHHx; Tissue engineering; Biomaterial

Introduction

Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx) is a natural biological polyester material that belongs to the polyhydroxyalkanoate (PHA) family (1,2). Compared with other, well-established PHA family members, poly(3-hydroxybutyrate) (PHB) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), PHBHHx has better biodegradability and mechanical properties. PHBHHx has excellent biocompatibility with a variety of cell types, including smooth muscle cells (3), fibroblasts (4), articular cartilage-derived chondrocytes (5,6), osteoblasts (7), and bone marrow cells (8). Additionally, PHBHHx possesses a low degree of crystallinity (2).

Polyhydoxyalkanoates are generally biodegradable and thermoprocessable and thus have been widely used in both conventional medical devices and tissue engineering. Because of its good biocompatibility, PHBHHx is attracting increasing attention as a cell scaffold for tissue engineering (2). Ye et al. (9) demonstrated that a three-dimensional PHB/PHBHHx scaffold seeded with differentiated human adipose-derived stem cells is capable of producing cartilage-like tissue after implantation into the subcutaneous layer of nude mice. PHBHHx scaffolds have been successfully applied in an *in vivo* tendon repair model, as evidenced by facilitation of tendon movement recovery and complete restoration of load bearing and function in recipient rats (10).

This review describes the two- and three-dimensional properties of PHBHHx and focuses on the improvement of its biological properties via surface modifications. Recent advances in the preparation and application of PHBHHx scaffolds are also discussed.

Scaffolds

Development and selection of a suitable scaffold is a critical aspect of tissue engineering. Scaffolds act not only as a physical support, but also as an artificial extracellular matrix (ECM) that contributes to the attachment, proliferation, and differentiation of seeded cells (11). An ideal scaffold material should meet the following criteria (12,13): *1*) nontoxicity of the polymer and its degradation products, *2*) excellent biocompatibility with seeded cells, *3*) good mechanical properties and flexibility, *4*) controlled biodegradability, *5*) easily formed as a porous structure, and *6*) appropriate thermoplastic properties.

Tissue engineering scaffolds can be roughly divided into natural and synthetic scaffolds. Synthetic scaffolds such as polyglycolic acid and polylactic acid (PLA) have been widely used as medical sutures, stents, and drug delivery carriers (14,15). Natural polymers including collagen, fibrin, chitosan, and PHAs are biocompatible and yield promising results when used as tissue engineering scaffolds (16-18).

Properties of PHBHHx scaffolds

Physical properties. Chen et al. (2) suggest that the changing PHA compositions allow favorable mechanical properties, biocompatibility, and degradation times within desirable time frames under specific physiological conditions (Table 1). From this table, we find that the melting temperature (T_m) and glass-transition temperature (T_g) of PHBHHx are dependent on the content of 3-hydroxyhexanoate (HHx), i.e., the higher the proportion of HHx, the lower the T_m and T_g values. Crystallinity of PHBHHx also decreases with increase in the content of HHx. In the composite system of PHB/PHBHHx, similar findings are observed. Chen et al. (2) also revealed that PHB/PHBHHx blended at a ratio of 1:1 has the maximum surface free energy, a key parameter affecting adhesion, distribution, and differentiation of seeded cells (2,19).

Toxicity of PHBHHx and its degradation products. PHBHHx is a copolyester of 3-hydroxybutyrate (HB) and HHx. PHB is not only an inert storage polymer found in the cytoplasm of many bacteria, but also a universal, interactive, solvating biopolymer involved in important physiological functions (20). Martin et al. (21) reported that, as the degradation products of PHBHHx, neither HB nor HHx are toxic, and even provide some therapeutic or nutritional benefits. It has been documented that HB facilitates neuronal survival and differentiation.

In vitro and in vivo PHBHHx biodegradation. Chen and Tong (22) evaluated, for the first time, the biodegradability of PHBHHx films *vs* PHB and Ecoflex and examined the effect of HHx content on PHBHHx degradation. They reported that, after 18 days of incubation, 40% P(HB-co-12%-HHx) and 20% PHB were degraded, while Ecoflex only lost 5% of its weight. Compared with PHB, P(HB-co-5%-HHx), and P(HB-co-20%-HHx), P(HB-co-12%-HHx) was degraded at a faster rate. Scanning electron microscopy analysis revealed that P(HB-co-12%-HHx) films had the most porous surface after degradation. These findings indicate that surface morphology plays an important role in the degradation of PHBHHx. P(HB-co-12%-HHx), with a low crystallinity and rough surface, had the highest degradation rate.

In vivo studies of PHBHHx and a PHBHHx/polyethylene glycol (PEG) blend implanted in rabbits for 6 months found that PHBHHx decreased in weight faster than PHB but slower than PLA (3,19). The degradation of PHBHHx was greatly improved by blending with PEG, but such blends led to a strong tissue response. Therefore, PHBHHx is a good biodegradable material and may be suitable for *in vivo* applications.

Biocompatibility properties. It has been reported that mouse fibroblast L929 cells plated on PHB/PHBHHx films grew better than those plated on PHB and PLA films, suggesting the PHB/PHBHHx blend is highly biocompatible (23). Similarly, Zhao et al. (24) demonstrated that chondrocyte cells grown on PHBHHx/PHB scaffolds possessed the physiological functions needed for effective cartilage tissue regeneration. However, the biocompatibility of PHAs is inadequate because of their hydrophobic properties (23). These findings suggest that improvement of the biocompatibility of PHBHHx is pivotal for its applications in tissue engineering. The methods commonly used to improve the biocompatibility of materials are discussed in detail in the third section of this review (Surface modification of PHBHHx for improved biocompatibility).

PHA family members for tissue engineering applications

PHAs

PHAs are a family of biodegradable polyesters synthesized by bacteria as intracellular carbon and energy storage compounds (2). More than 150 kinds of monomeric PHAs have been reported. Although PHAs have the potential to be used as tissue engineering biomaterials, only a few of them, such as PHB, PHBV, and PHBHHx, have been produced industrially (25). PHB is one of the most thoroughly researched members of the PHA family and has good biocompatibility for adrenocortical cells, osteoblasts, epithelial cells, fibroblasts, endothelium cells, and isolated hepatocytes (26). However, it is highly brittle and degrades slowly, and

Table 1. Physical properties of various polyhydroxyalkanoates compared with conventional plastics.

Samples	T_m (°C)	T_g (°C)	Tensile strength (Mpa)	Elongation at break (%)
PHB	177	4	43	5
P(HB-co-10% HV)	150	–	25	20
P(HB-co-20% HV)	135	–	20	100
P(HB-co-10% HHx)	127	–1	21	400
P(HB-co-17% HHx)	120	–2	20	850
Polypropylene	170	–	34	400
Polystrene	110	–	50	–

T_m: melting temperature; T_g: glass-transition temperature; HB: 3-hydroxybutyrate; HV: 3-hydroxyvalerate; HHx: 3-hydroxyhexanoate.

both of these properties have limited its applications (27). Another PHA family member, PHBV, can be produced as fibers that have been shown to promote reepithelialization (28). After surface modification, PHB and PHBV are able to promote osseointegration (29). However, long-term exposure to PHB and PHBV may induce an inflammatory response.

Brief introduction of PHBHHx

The new PHA family member, PHBHHx, is considered a very promising tissue engineering material with adjustable mechanical properties and good biocompatibility and biodegradability (3). Experimental studies have indicated that PHBHHx has high biocompatibility for a wide range of cell types such as smooth muscle cells, fibroblasts, chondrocytes, osteoblasts, and bone marrow cells (5,30-33). The PHBHHx films were created using solvent-casting, electrospinning, and compression molding methods, and the behavior of human mesenchymal stem cells (hMSCs) grown on these films has been investigated (34). hMSCs cultured on highly porous electrospun PHBHHx films have better cell viability than those cultured on compression-molded films.

PHBHHx particles have been used as drug delivery systems in targeted anticancer therapy (35-37). PHBHHx combined with folic acid and loaded with etoposide (a cell cycle-specific anticancer drug) allows improvement of the targeted delivery of drugs to tumors. In another application, PHBHHx nanoparticles loaded with an insulin-phospholipid complex have been used in a long-acting release formulation of insulin. However, practical applications of PHBHHx have been limited because of some disadvantages such as high hydrophobicity and low crystallization rate. To overcome these limitations, many attempts have been made to modify the physicochemical properties of PHBHHx and to develop new formulations.

Surface modification of PHBHHx for improved biocompatibility

Surface treatment

Surface modification is an effective way to improve the hydrophilic properties of PHAs. Different strategies, including ultraviolet treatment, plasma treatment, grafting techniques, and surface hydrolysis (38-40), have been applied to increase the hydrophilicity of polymer materials. These treatments can be used not only to increase the hydrophilicity of the surface material, but also to introduce biologically active molecules to polymer surfaces, thus modifying the biocompatibility of materials. Alkali and lipase treatments are very effective to improve the wetting and adhesion properties of polymer surfaces (32,41). It has been demonstrated that NaOH treatment enhances the attachment of cells to PHBHHx films (42). However, these surface modification techniques do have some disadvantages. For example, grafting makes the surface of the material very unstable because of the very weak interaction between the bulk polymer and the bioactive molecules. Direct ultraviolet radiation may change the mechanical properties of polymer films and make them very brittle. Plasma treatment may only temporarily enhance the hydrophilicity of PHAs.

Blending with other materials

The blending of materials with different properties is a commonly used approach to offset individual defects. Mei et al. (43) evaluated the biocompatibility of PHBHHx modified by silk fibroin (SF). They found that the water contact angle on the PHBHHx/SF was smaller than that on the pure PHBHHx scaffold, and the number of cells attached to the surface of PHBHHx/SF films was larger than on the control films, indicating an improvement in hydrophilicity and biocompatibility.

Additionally, PHA family members may be blended with each other. Scaffolds made from PHBHHx and PHB at a ratio of 1:1 have a higher surface free energy and are more effective for chondrocyte adhesion than scaffolds made of PHBHHx alone (19). In another study, adipose-derived mesenchymal stem cells inoculated on PHB/PHBHHx scaffolds and implanted into animals were found to generate cartilage-like tissues 14 days after implantation (2).

Biomodification with amphiphilic proteins

Recently, biomodification of polymer surfaces has emerged as a novel approach to improve their wetting and adhesion properties. Several amphiphilic proteins have been identified to be located on the surface of *in vivo* PHA granules including PhaC synthase, poly-beta-hydroxybutyrate depolymerase (PhaZ), and granule-associated protein (PhaP) (44). Previous studies showed that PhaP fusion proteins are able to bind to natural PHA granules, allowing for the development of low-cost approaches for protein purification, specific drug delivery, and cell sorting. Arginine-glycine-aspartate (RGD) tripeptide is normally involved in cell binding to a number of ECM glycoproteins by interaction with integrins (45). The PhaP-RGD fusion protein, when used to coat PHBHHx, has been shown to enhance cell adhesion, spreading, migration, and proliferation (31). Similarly, You et al. (5) showed that PhaP-RGD-coated PHBHHx scaffolds facilitate chondrogenic differentiation of human bone marrow mesenchymal stem cells inoculated onto them.

Scaffold fabrication

There are currently several methods available for fabrication of PHBHHx scaffolds, including solution casting, electrospinning, phase separation, solvent casting, fiber bonding and particulate leaching, solid freeform fabrication, and particle sintering (46,47).

Solvent evaporation method

Solvent evaporation is a simple method and the most

commonly used to fabricate PHBHHx films (34). In detail, a PHBHHx solution is prepared by dissolving purified PHBHHx in chloroform, which is then evenly distributed into glass dishes. The chloroform is allowed to evaporate at room temperature, resulting in the formation of PHBHHx films on the Petri dishes. Vacuum drying is generally applied to eliminate the residual solvent, because of its cytotoxicity.

Phase separation and freeze-drying

Freeze-drying is commonly used to prepare three-dimensional scaffolds (33). The principle involved is that the polymer solution, emulsion, or hydrogel is frozen to induce phase separation, and the solvent is then removed by vacuum freeze-drying to form a porous scaffold. The pore morphology, mechanical properties, biocompatibility, and degradability of the scaffold are affected by the concentration of the polymer solution and the phase separation temperature, the quenching temperature, and the types of polymer and solvent.

Wang et al. (48) fabricated PHBHHx scaffolds by thermally induced phase separation. In this process, polymers are dissolved in 1,4-dioxane under vigorous agitation for 60 min at 65°C and the mixture is then kept frozen at –80°C for 2 h. After removal of the solvent via freeze-drying, porous scaffolds are obtained, with a pore size of approximately 60-100 μm in diameter and a porosity of 93 ± 1.4%. Such porous three-dimensional scaffolds provide additional space for seeded cells to grow.

Electrospinning techniques

Electrospinning techniques have been used to create fibrous scaffolds that mimic the natural ECM for study of *in vivo*-like phenotypic shape and gene expression (49). Interestingly, the orientation of electrospun fibers is able to provide contact guidance for the attached cells, and has an impact on their morphology and even their differentiation capacity. It has been reported that aligned electrospun fibers promote Schwann cell maturation, neurite outgrowth, vascular endothelial cell growth, and myotube formation (6). The regular alignment of fibers leads to enhanced osteogenic responses in MSCs compared with the fibers having a randomly networked architecture.

The application of electrospinning techniques in tissue engineering has been reviewed (50). Three-dimensional fibrous and nanofibrous PHBHHx-based scaffolds that have been successfully produced by electrospinning show an osteoinductive potential. However, it is difficult to control the porosity of scaffolds produced using this technique.

Particle leaching

Highly porous scaffolds can be fabricated by particle leaching using sodium chloride (NaCl) particles as a porogen (51). Sun et al. (51) demonstrated that the porosity of scaffolds is almost independent of the porogen size, but instead is dependent on the amount of porogen. The maximum porosity of scaffolds can reach about 85%, when the weight ratio of porogen to polymers is 9:1. The fabrication process began with dissolving 2 g PHBHHx powder in 20 mL chloroform in a 50-mL conical flask and then 0.04 g NaCl crystals was suspended in the PHBHHx-chloroform solution and mixed by shaking. Tube construction was carried out using a 2.5-mm diameter stainless steel mandrill, which was dipped into the homogenized PHBHHx/NaCl solution for 1 s, followed by solvent evaporation in a flow hood for 2 min. This process was repeated five times, after which the mandrill was fixed vertically for 60 min to allow the solvent to fully evaporate and the polymer to become rigid. The polymer tube was carefully removed by hand and immersed in deionized H_2O overnight to introduce porosity through NaCl crystal dissolution.

Applications of PHBHHx-based biopolyesters

In recent years, PHBHHx-based biopolyesters have been developed for various potential medical applications, especially as tissue engineering and drug delivery materials.

PHBHHx as a material for bone and cartilage tissue engineering

Cells for bone and cartilage regeneration have been evaluated for their proliferative and differentiative responses when inoculated into PHBHHx-based scaffolds (32,52). It has been reported that PHBHHx scaffolds increase both chondrocyte proliferation and protein secretion in rabbit articular cartilage cells seeded onto them (53). Chondrocyte proliferation and synthesis of glycosaminoglycans (GAGs) and type II collagen are generally required for successful cartilage regeneration.

Compared to those seeded on PLA and PHB, rabbit bone marrow cells inoculated on three-dimensional PHBHHx scaffolds show more differentiation into osteoblast-like cells, with a round cell shape, high alkaline phosphatase activity, strong calcium deposition, and fibrillar collagen synthesis. This finding suggests that PHBHHx is a suitable biomaterial for promotion of osteoblast attachment, proliferation, and differentiation.

Chondrocytes isolated from rabbit articular cartilage were inoculated onto numerous PHBHHx/PHB porous scaffolds (54). Total collagen content in all scaffolds containing PHBHHx increased with the duration of incubation. Moreover, the use of PHB/PHBHHx at a ratio of 1:2 in scaffolds resulted in a significant increase in the production of GAG, a major composition of ECM, after 7 days of culture, compared with scaffolds having different component ratios. Therefore, the proportion of PHBHHx in the PHB/PHBHHx composite system is of importance for the promotion of ECM synthesis by articular cartilage

chondrocytes. The inoculated cells grew better on the scaffolds consisting of PHBHHx/PHB at the ratios of 2:1 and 1:2 than on those having a 1:1 ratio.

Nerve and brain tissue repair

PHAs have shown good potential as medical implant biomaterials. Neural stem cells (NSCs) grown on/in PHA scaffolds may be useful for repairing central nervous system (CNS) injury (46). Among the PHA family members, PHBHHx appears to have the strongest potential to promote NSC differentiation into neurons. It has been suggested that PHBHHx nanofiber scaffolds that promote NSC growth and differentiation could be developed for treating CNS defects.

Porous nerve conduits with either uniform or nonuniform wall porosity were prepared using a particle leaching method. The conduits were used to bridge a 10-mm defect in the sciatic nerve of rats (55). PHBHHx nerve conduits prepared in this study showed satisfactory mechanical strength and biodegradability. Histological assessment together with transmission electron micros-copy showed that the performance of conduits with nonuniform wall porosity was similar to that of nerve autografts in promoting functional recovery, but better than the conduits with uniform wall porosity. Thus, PHBHHx-based conduits may represent a promising alternative for peripheral nerve damage repair.

Soft tissue repair and smooth muscle engineering

Considering that its strength and elastic properties can be adjusted by changing its monomer contents, PHBHHx could be tailored to meet the requirements for regenerat-ing both bone and soft tissues. PHBHHx, with its excellent mechanical and thermal properties, has been used as a scaffold for tissue-engineered cardiovascular products (19). Qu et al. (56) prepared and modified PHBHHx by ammonia-plasma treatment and fibronectin coating and then inoculated human umbilical vein endothelial cells (HUVECs) and rabbit aorta smooth muscle cells on the surface of the modified PHBHHx scaffold (PFn-PHBHHx). They found that HUVECs proliferated more on the PFn-PHBHHx than the PHBHHx surface and formed a confluent monolayer 3 days after seeding. This finding suggests that PFn-PHBHHx may be a good material as a luminal surface of vascular grafts.

Due to good elasticity and strength, PHBHHx may be a promising candidate material for the generation of artificial esophagus. Indeed, some studies using PHBHHx as artificial esophagus in dogs indicated that PHBHHx can stimulate regeneration of removed esophagus tissue (2).

PHBHHx as biodegradable drug carriers

PHBHHx particles have been used for drug delivery because of their biodegradability and nontoxic, nonimmu-nogenic degradation products. For example, PHBHHx nanoparticles and micron particles were used as drug delivery carriers for the controlled release of 5-fluorouracil (57). In combination with folic acid, PHBHHx nanoparti-cles have been used to deliver etoposide, a cell cycle specific anticancer drug, to tumors (58). Insulin phospho-lipid complex loaded biodegradable PHBHHx nanoparti-cles are effective in the delivery of long-acting insulin (58).

Conclusions

PHBHHx offers great practical potential as a tissue engineering scaffold material. Numerous production techniques are available to modify or improve the physicochemical and biocompatible properties of PHBHHx. With novel composite material generation coupled with technical innovations, PHBHHx-based scaf-folds may be of clinical benefit in regenerating a variety of tissues.

Acknowledgments

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