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Review Article

Poly hydroxyalkanoates (PHA): Role in bone scaffolds

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ABSTRACT

Polyhydroxyalkanoates (PHA) are prokaryotic macromolecules accumulated within the cytoplasm as granules. Due to their suitable mechanical properties, biocompatibility, degradation time, ability to be blended, surface modified, and form copolymers, it is widely used in medical devices and as scaffolds in bone tissue engineering. This review describes in brief the production and extraction sources, physico-chemical characteristic, mechanical properties, degradation rate and applications of various PHAs and its copolymers with special emphasis to its role as scaffolds in bone tissue engineering.

1. Introduction

Polyhydroxyalkanoates (PHA) are biocompatible and biodegradable polyesters accumulated within the cytoplasm of prokaryotic cells as water insoluble granules. PHA extracted from these granules exhibits good tensile strength, thermoplasticity and elastomeric nature. These properties of PHA and its polymers are comparable to bone and thus qualify them to be used as scaffolds for bone engineering.

2. PHAs

Polyhydroxyalkanoates (PHAs) are prokaryotic storage macromolecules, accumulated intracellularly as energy storage materials by various microorganisms under unbalanced specific growth conditions i.e. when the growth medium contains excess carbon (C), or low concentrations of nitrogen (N), phosphorus, or magnesium. These granules are often known as “carbonosomes” and are around 0.2–0.4 μm in diameter and contribute to more than 90% of the cell mass. Dyes like Sudan Black B and oxazine dyes like Nile Blue A or Nile Red can be used to stain the accumulated PHA in the cells.[1]

PHAs are aptly referred as ‘green plastics’ due to their positive social and environmental impact in production and recycling. With recent advancements, they can even be developed from non-PHAs producing strains with no toxins.[2]

3. PHYSIO-CHEMICAL characteristics

PHAs can be classified on the basis of the number of carbon atoms in the monomer incorporated into the polymers by their chain length; as short-chain-length PHAs (SCL-PHAs; 1-5-carbon atom monomer), medium-chain-length (MCL-PHA) with C6–C14 and long-chain-length (LCL-PHA) with > C14 monomers. The physicochemical properties of the PHAs may vary depending on their composition. While the SCL-PHA has properties similar to conventional plastics polyethylene or polypropylene, MCL-PHA has properties close to elastomers and rubbers. The SCL-PHA has high (60–80%) crystallinity, is stiff and brittle, while the MCL-PHA has low crystallinity, low glass transition temperature, low tensile strength and high elongation at break.[3]

The composition of PHAs varies depending upon the organism and their carbon substrate. Its molecular mass ranges between 200 and 300 kDa depending on the metabolic capability of the bacteria.[4]

The chief characteristics of PHA include non-antigenicity, biocompatibility, good tensile strength, enantiomeric purity, thermoplasticity and elastomericity.[5] However, PHAs differ from other currently available biodegradable plastics due to moisture resistance, and water insolubility.[6]

4. Mechanical properties

The mechanical properties depend upon the monomeric composition of PHA, its chain length and the distance between R-group and ester linkage. Poly-3-hydroxybutyrate (P3HB) is SCL-PHA with 3-hydroxybutanoic acids units, is versatile and can be extruded, molded, spun into fibers, made into films or blended. It is stiff with a tensile modulus (3.5 GPa), tensile strength (40 MPa) and an elongation at break (6%) and shows good oxygen impermeability.[7]

Another member, poly 4-hydroxybutyrate (P4HB) is strong thermoplastic, malleable material, with tensile strength equivalent to

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polyethylene, flexible with 100% elongation at break, tensile modulus (0.15 GPa), tensile strength (104 MPa). PHB also presents piezoelectric properties, similar to natural bone.[5] Though both are SCL-PHA and have equal number of carbon in their chains, they differ in R group positions, leading to difference in their 3D structure, polymer crystallinity and mechanical properties.[8]

Integration of 3-hydroxyvalerate (HV) units in PHB results in fabrication of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) or copolymer PHBV, with a lower melting point and crystallinity, tougher, better flexibility, and tolerance to thermal processing.[9] PHBV is water insoluble and moisture resistant, does not degrade under normal conditions of storage, and is indefinitely stable in air.

Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx) has lower crystallinity, broader processing window and higher elasticity compared with PHB and PHBV due to its long alkyl chain. It also possesses piezoelectric behavior and cytocompatibility when cultured with stem cells.[10,11]

Currently PHB, PHBV, P4HB, PHBHHx, copolymers of 3HB and 4-hydroxybutyrate (P3HB4HB), and poly-3-hydroxyoctanoate (PHO) are being used for tissue engineering.[12]

5. PHA production and extraction

PHA polymer, PHB/P3HB (Poly-3-hydroxybutyrate) was the first isolated and characterized in 1925 by **Maurice Lemoigne.** The main constraint lies in its high cost of production. However, currently several ways are being used to make the process cheaper, for example, using suitable bacterial strains and inexpensive carbon sources like those described in Table 1. Another cause of increased cost of production is the intracellular location of PHA along with complexity of the procedure of extraction. The methods employed for PHA production and extraction are described in brief in Table 2.

Porous scaffolds can be produced by thermally induced phase separation, evaporation, freeze-drying, solid-free fabrication, 3D printing, selective laser sintering, solvent casting, foam-coating, and many other techniques[13]

6. Degradation of PHA

PHAs are biodegradable although the rate of degradation observed is very slow and is primarily microbial ie. PHAs gets converted into CO2 and energy by microorganisms such as bacteria, fungi, and algae. Following degradation, the products pass though the cell wall and are metabolized. Tokiwa Y et al. (2004) demonstrated that PHB and PHBHV degrades *in vivo* to D-3- hydroxybutyrate (3HB), a common human blood constituent. Hence PHAs have an edge over other biodegradable polymers as are hydrolyzable, being degraded to soluble monomers without the help of other organisms.[5] The hydrolysis products, 3HB and 4HB monomers, are natural metabolites that exist in brain, lung, heart, liver, kidney, muscle and expired as carbon dioxide from our body.[14] Biodegradability of polymers is inversely related to melting point, crystallinity and molecular weight of the polymer[15]

Table 1
Source of Carbohydrates for low cost PHA production.

S no.	Chief content	Source
1	Carbohydrates	Sugar beet molasses Starch and starch hydrosylates Maltose and Lactose from whey Cellulose hydrosylates Reject fiber wastes from the paper industry after hydrolysis
2	Alcohols	Wastes from biodiesel production Methanol and Glycerol
3	Fats and oils	Plant and animal wastes
4	Organic acids	Lactic acid from dairy industry

Table 2
Methods employed for PHA extraction.

S No.	Method of extraction	Description	Advantage/Disadvantage	Reference
1	Solvent extraction	Chloroform is used as a solvent	High purity, without degradation of the PHA molecules, but the potential toxicity of the solvents is the problem	Yellore V & Desai A (1998)
2	Sodium hypochlorite Dissolution	Cellular mass is submerged in a sodium hypochlorite solution causing cell lysis followed by centrifugation to obtain PHA granules	Causes degradation of PHA	Ward A C & Dawes E A (1973), Heinrich D et al. (2012), Hahn et al. (1993)
3	Enzymatic degradation	Use of enzymes like proteases, trypsin bromelain, cellulose and lysozymes	High PHA yield without degradation, but these methods require heat treatment to break the cells.	Kanmani P et al. (2016) Choi and Lee (1999)
4	Centrifugal fractionation	The crop is pretreated with hexane for eliminating molecular lipids and oils, and then treated by 40%water/60% ethanol to eliminate soluble compounds like sugar	Up to 85% of PHA with purity higher than 95% can be obtained from continuous centrifugal fractionation	Chee JY et al., 2010
5	Electrospinning	To produce nanometeric to micrometric polymeric fibers	Helpful for tissue engineering scaffold fabrication	Sill TJ et al., 2008 Smith IO et al., 2009

7. Applications

Mechanical properties, biocompatibility, and degradation times of PHA make it suitable for application in tissue engineering. The fact that PHAs can be blended, surface modified, and form copolymers, enhances its application in medical devices and bone tissue engineering.

PHA, PHB, PHBV, P4HB, its composites and copolymers of 3-hydroxybutyrate, PHBHHx, and PHO are used to develop sutures, patches, slings, pins, barriers, stents, medical devices for guided tissue regeneration, cartilage or tendon repair, nerve guides, bone scaffolds, and wound dressings[16]

8. PHA as biomaterial for bone scaffolds

PHA and its composites possess several characteristics to qualify as biomaterial for scaffolds. When compared to other polymers like polylactide-*co*-glycolid (PLGA), polyglycolic acid (PGA), and polylactic acid (PLA), PHB scaffolds local pH during degradation remains unchanged, making them well tolerated by immune system.[17]

Blend of P(HB-co-8%HV)/hydroxyapatite (30% w/w) has a mechanical compressive strength as of human bones (62 MPa), evokes lower inflammatory response and causes higher mineralization.[18] Shishatskaya et al. (2004) and Volova et al. (2003) implanted rats with PHA sutures and observed them in long-term studies to find PHA threads remain active throughout and do not show any adverse effects.[19]

Ellis et al. prepared laser-perforated biodegradable scaffold films of PHBHV and observed that the human keratinocytes attached and grew on film surface, penetrated pores and reached the damaged tissue. The decreased crystallinity at pore edges enabled faster cell adhesion, growth and migration[20]

Puppi et al. blended PHBHHx with PCL by computer-aided wet-spinning to avoid any adverse effect on the PHA molecular mass and provide pre-defined macro- and micro-porosity in the scaffold. They observed successful adhesion and proliferation of pre-osteoblast cells.[21] There are several successful reports on PHB and PHBV *in vitro* and *in vivo*, for bone tissue regeneration approaches.[22] PHBHHx has been used in the form of micro-grooved membrane,[23] aligned nanofibers[23] or carbon nanotubes-loaded composite materials[24] and has shown to support human mesenchymal stem cells in osteogenesis.

Various Additive manufacturing techniques like stereolithography and fused deposition modelling were used to develop a predefined scaffold shape and porosity at macro-to micrometric scale[25] Despite the promising results, the narrow melt processing temperature window of this technique hindered its application in production of 3D PHA porous scaffolds. Now a days, a hybrid technique, computer-aided wet-spinning (CAWS), is gaining attention. This technique involves processing of PHBHHx into 3D scaffolds wherein, a computer-controlled deposition of a solidifying polymeric fiber extruded directly into a coagulation bath occurs.[26]

9. PHA blends

PHB has been explored extensively as a scaffold biomaterial and approved by FDA. However, PHB is highly crystalline with a brittle nature, a relatively long degradation time and hydrophobic in nature thus limiting its application.[27,28] A number of studies have attempted to modify the material properties of PHB through blending.[29,30] A 50: 50 (w/w) blend of PHB with PHBV has shown better support for attachment and proliferation of human osteoblast cells.[31] Daranarong D et al. (2014) fabricated electrospun nanofibrous scaffolds using fibrous membranes of PHB with polyL-lactide-co–caprolactone (PLCL) and observed them to be more hydrophilic (< 120°) with lower tensile strength, increased extension at break, greater adhesion. This scaffold showed enhanced proliferation and mitochondrial activity of cells. PLCL/PHB nanofibrous membranes have also shown to promote cell cycle progression and reduce the necrosis.[32]

Electrospun PHB/cellulose acetate (CA) blended nanofiber scaffolds altered the crystallization of PHB by formation of hydrogen bonds and the CA content increased the glass transition temperature. Percentage increase in PHB increased the tensile strength, yield strength and elongation at break of the blended nanofiber scaffolds and decreased the water contact angle. *In vitro* degradation rate of blended nanofiber scaffolds was much higher, and the cells showed better biocompatibility and were capable of cell adhesion and proliferation.[33]

When blends of PHBHHx and polyD,L-lactic acid (PDLLA) were fabricated into fibrous membranes by electrospinning, the mechanical properties of the electrospun fibrous membranes depended on the orientation of fibers. They had higher elongation; tensile strength and modulus. As PDLLA increased, the electrospun fibrous membranes showed higher elongation and lower tensile modulus. PDLLA degraded faster than PHBHHx.[34]

When PHA was used to prepare open porous microspheres of 300–360 μm diameter, it could be used as injectable carrier harbouring proliferating stem cells. In contrast to PLA, PHA presented a high *in vitro* cell adhesion of 93.4% with surface pores of 10–60 μm and interconnected passages of 8.8 μm average size, continuous proliferation for 10 days, improved differentiation, a stronger osteoblast-regeneration and protection of cells against stresses during injection, which allowed proliferation and migration of more living cells to the damaged tissues.[35]

When hydrophobic PHBHHx scaffolds were coated with a PHA granule binding protein fused with RGD peptide (PHAp-RGD), it enabled homogeneous spread of cells for better adhesion, proliferation and chondrogenic differentiation, more production of ECM and significantly more cartilage-specific sulphated glycosaminoglycans (sGAG) and total collagen content.[36]

10. Conclusion

The application of PHA as scaffold in bone tissue regeneration is quite recent, and has a lot of potential due to its biocompatibility. However, their major disadvantage is that mostly they are produced by microorganisms in stressful environments (lacking nitrogen, oxygen, magnesium phosphate or sulphate), that limits their availability and hence increases the cost of fabrication. One of these PHB has a high molar mass, a decreased biodegradation rate and low electronic density because of which it cannot absorb enough photons to produce high-contrast images and thus cannot be seen radiographically.

Declaration of competing interest

Authors have no conflict of interest to disclose.

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ICMR Bioengineering: Biomaterial for designing of mandibular bony scaffold: Evaluation of polymer-ceramic nano composite scaffold with BMP for critical size mandibular defect.(5/3/8/290/2015-ITR: 28,09,800)

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