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Activation of polyhydroxyalkanoates: functionalization and modification

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1. ABSTRACT

Polyhydroxyalkanoates (PHAs) serve numerous bacteria as storage compounds. It is generally believed that under unbalanced growth conditions, n-hydroxyalkanoates are synthesized inside the bacterial cells, polymerized to polyesters, and densely packed in granules. In the absence of extracellular carbon, the internally stored PHAs are depolymerized and consequently metabolized to enable cell maintenance and reproduction. However, some bacteria exhibit growth associated production and degradation of PHAs as part of the cell sustainment. This natural production and degradation cycle indicates that PHAs possess biodegradability and may have biocompatibility properties. Since the discovery that some bacteria can incorporate 3-hydroxyalkanoates bearing functional groups from related substrates, research has led to structural diversification of PHAs by biosynthesis and chemical modifications. A commonly applied route for tailoring PHAs is their *in situ* functionalization by biosynthetically producing side chains with terminal double bonds followed by chemistry. Non-functionalized PHAs can also be activated by surface modification techniques. The resulting tailor-made structural and material properties have positioned polyhydroxyalkanoates well to contribute to the manufacturing of second and third generation biomaterials.

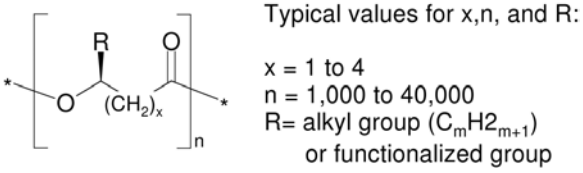

Figure 1. General structural formula of polyhydroxyalkanoates

2. INTRODUCTION

Functionalization describes the introduction of chemical functional groups to polymeric materials. With respect to synthetic polymers, this process mainly includes the manipulation of surfaces by different coating techniques to tailor their surface properties and to meet specific manufacturing needs. The advantage of having functionalities in biological polymers and macromolecules was realized early as their functional groups can be used to control processibility, mechanical properties, or solubility by chemical modifications (1). Recently biomaterials have been designed with applications that exceed those of conventional biopolymers like proteins, cellulose, and polyribonucleic acids. Consequently biomaterials may soon replace conventional, petroleum-based plastics in many areas including regenerative medicine. Furthermore, biomaterials may be converted into smart or multi-functionalized biomaterials, which respond to specific stimuli and can therefore fulfill multiple tasks (2, 3). Despite rapid progress in this field, the functionalization itself remains a challenge as it may require much energy, complex equipment, and reactive chemical reagents.

In comparison to other biomaterials, polyhydroxyalkanoates (PHAs) are a family of biodegradable and biocompatible polymers that have the potential to facilitate their functionalization as it may be integrated into the biotechnological production process. The broad substrate specificity of some PHA synthases enables the fermentative production of PHAs bearing functional groups in their side chains. As a result, these polymers can easily be chemically modified to improve their properties or to covalently bind other molecules and polymers. Composite materials that have a core composed of PHAs are of significant interest in tissue engineering and controlled drug release applications (4-8). To date the fabrication of composite materials including PHAs is typically achieved by blending them with other polymers (9-12).

Polyhydroxyalkanoates may be subjected to chemical and physical modification steps to improve their thermo-mechanical and surface properties. A number of such modifications can be performed to make PHAs more valuable and to expand their application spectrum. This review aims to introduce the reader to functionalized polyhydroxyalkanoates from their discovery to the most recent developments in production. Moreover, natural, chemical, and physical modification techniques to activate functionalized and non-functionalized PHAs are presented. Since PHAs qualify for fine applications, they are also briefly discussed as potential candidates for tissue engineering materials.

3. POLYHYDROXYALKANOATES AND THEIR PROPERTIES

The primary functions of polyhydroxyalkanoates are to act as internal carbon and energy storage (13-16). PHAs are water-insoluble polyesters intracellularly produced, often promoted by unbalanced growth conditions, and stored by a wide range of bacteria, some yeasts, and a few transgenic plants. Bacteria, the predominant microorganisms for polyhydroxyalkanoic acid production, synthesize PHAs especially in the presence of excess carbon source and under limiting nutrient conditions. Once the limiting nutrient is supplied, the carbon storage in form of PHA is degraded and used for growth. The type of microorganism and the growth conditions applied determine the molecular weight of the PHAs and these usually range from 200 kDa to 3 MDa (14, 17). Choi and Lee reported the production of supra molecular weight poly(3-hydroxybutyrate) (PHB) as high as 22 MDa by metabolically engineered *Escherichia coli* cells (18). PHB, illustrated in Figure 2, is the most common and known member of these polyesters with a methyl group at the third carbon atom on the polymer backbone.

PHA forming units can be combined to form an endless variety of different copolymers. To date more than 150 types of biologically produced PHAs have been reported (19-21), a value that already exceeds the number of PHAs that are available from chemical synthesis (16). Poly(3-hydroxyalkanoates) are classified by the length of their monomeric units (22, 23):

1. Short-chain-length-3-hydroxyalkanoates (scl-3HA) with 3-5 carbon atoms.
2. Medium-chain-length-3-hydroxyalkanoates (mcl-3HA) with 6-14 carbon atoms.
3. Long-chain-length-3-hydroxyalkanoates (lcl-3HA) with 15 carbon atoms and more.

Poly(3-hydroxyalkanoates) may contain any combination of the monomeric unit classes listed above, including copolymers with scl- and lcl-3HA units (scl/lcl-PHAs), which were recently produced (24). The most common PHAs, however, are scl-PHAs and mcl-PHAs. The side-chain length determines the thermo-mechanical properties of the polymer and plays a crucial role in selecting the right PHA for the desired application.

3.1. Biocompatibility

Arguably the most important consideration when employing extrinsic materials for medical applications is their biocompatibility. Potential candidates are typically excluded if they cause toxic reactions. Materials that are chosen to interact with living tissue, therefore, have to undergo intensive *in vitro* and *in vivo* studies to ensure their capability to function properly. Williams defined biocompatibility as

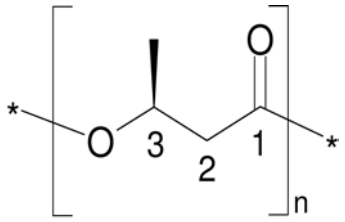

Figure 2. Poly(3-hydroxybutyrate) (PHB)

"the ability of a material to perform with an appropriate host response in a specific application" (25).

Biocompatibility thus describes tissue-material interactions. There are three factors, which the biological response of a material depends on (26):

1. The properties of the material.
2. The characteristics of the host.
3. The functional demands on the material.

Only a combination of these factors is sufficient to make a statement on the biocompatibility of the application. This conclusion makes general predictions about PHA tolerance difficult, especially since most information is currently only available for PHB and PHBV (27). However, present studies related to these polymers allow for a basic evaluation of the biological response to PHAs in general.

The monomeric component of PHB, 3-hydroxybutyrate (3HB), is a common metabolite found in all higher organisms (27). The blood concentration of this hydroxy acid was reported to be between 0.03 and 0.1 g/L in healthy adults (cited in 28). The monomeric unit of poly(4-hydroxybutyrate) (P4HB) is also widely distributed in mammals (29). These two PHA components have been studied intensively for applications in human and animal medicine (30-35). Natural occurrence of these monomeric units suggests that their polymeric forms could induce positive biological response. In addition, Reusch and co-workers reported PHB to be a ubiquitous component of the cellular membranes of animals (36). As such, these findings serve as evidence for desired biocompatibility of implanted PHB (37).

In vitro cell culture and *in vivo* tissue responses have been studied with different PHAs in the past. These topics have been the subject of several reviews recently (26, 27, 38). In general, PHAs were found to show high biocompatibility in cell cultures and to be well tolerated *in vivo*.

3.2. Biodegradation

The inescapable predicted shortage of crude oil has led to focused research aimed at reducing the use of petroleum-based products. In the search for renewable energy and feedstock, the term "green" has become a synonym for alternative products that are environmentally friendly. Consequently, "green" plastics may play an important role in achieving a sustainable future.

Green plastics are those which become degraded to environmentally acceptable products (39). This process, known as biodegradation, defines the decomposition of material by microorganisms. It is rational to suggest that any biologically produced material is also biodegradable. However, this feature is not exclusively reserved to biopolymers. There are synthetic polymers, which successfully mimic the ability of being biodegradable. Thus, not only the source but also the texture, conformation, and co-monomeric composition are important factors for biodegradation (40). Biodegradable plastics can be divided into three groups (17):

1. Chemically synthesized polymers.
2. Starch-based plastics.
3. Polyhydroxyalkanoates.

The first group includes polyglycolic acid (PGA), polylactic acid (PLA), polyvinyl alcohol, and poly(ethylene oxide) (PEO) (41). It should be noted that the biomedical potential of PGA and PLA was reported after PHB (26, 42, 43). However, these synthetic polymers have been the subject of significant industrial research, leading to medical applications primarily due to their "industrial" advantage: potential for cheap production costs via chemical synthesis. Hence, many different synthetic polymers have become trademarked, for example PLA, manufactured as LACEA by Mitsui Chemicals Inc. (Tokyo, Japan) (44). Additional commercialized synthetic polymers from this group also include (40):

1. Ecoflex by BASF AG (Ludwigshafen, Germany). Aliphatic-aromatic copolyester consisting of terephtalic acid, 1,4-butanediol, and adipic acid (45).
2. Biomax by DuPont (Wilmington, DE, USA). Aliphatic-aromatic copolyester consisting of ethylene glycol, diethylene glycol, terephtalic acid, adipic acid, and sulfo isophtalic acid (46).
3. Bionelle by Showa Highpolymer Co., (Tokyo, Japan). Poly(tetra- methylene succinate) (47).

Many starch-based plastics are only partially biodegradable. The starch functions as filler and crosslinking agent. The starch component of the resulting blend (for instance starch-polyethylene) is easily degraded by microorganisms; however, the polyethylene component can remain in the environment for a significant period of time (17, 48). An exception can be found when starch is blended with petroleum-derived polycaprolactone (PCL), which belongs to the family of Mater-Bi^{TM} starch-based polymers (Novamont, Italy). This hybrid of a natural and a synthetic polymer is fully compostable (49).

Polyhydroxyalkanoates, belonging to the third group, are 100% biodegradable plastics due to the ideal biosynthesis-biodegradation cycle they undergo (39). The resulting degradation products are water and carbon dioxide under aerobic conditions and methane and CO_2 under anaerobic conditions (50, 51).

The enzymes that are responsible for the depolymerization of polyhydroxyalkanoic acids are called

Table 1. Thermo-mechanical properties of selected PHAs in comparison to polypropylene and polystyrene

Polymer	Melting temperature T_m [C][1]	Glass transition temperature T_g [C][1]	Tensile strength [MPa]	Elongation at break [%]
PHB	177	4	43	5
PHBV (90/10) [2]	150	2	25	20
PHBO (92/8) [2, 3]	125	-4	ND	ND
PHBO (87/13) [2, 3]	108	-8	ND	ND
PHOHxD (86/12/2) [4]	62	-33	9	380
PHOHx (88/12) [2]	61	-35	9	380
PHOU (95/5) [2]	62	-35	ND	ND
PHOU (66/34) [2]	54	-40	ND	ND
P4HB	60	-50	104	1000
PHB4B (84/16) [2]	152	-8	26	444
Polypropylene	170	-10	34	400
Polystyrene	110	21	50	ND

Adapted from (17, 27, 62, 73-75). [1] degrees Celsius. [2] PHXY (m/n) = m mol% of X and n mol% of Y. [3] 3HHx was likely present as well. [4] PHXYZ (m/n/o) = m mol% of X, n mol% of Y and o mol% of Z. Note: the thermo-mechanical properties of PHAs also depend on their molecular weight distribution and average. Thus, author's results do not always agree 100%. However, the values summarized in this table can be seen as indicators. ND = no data.

PHA depolymerases, which can be found both inside and on the outside of cells. Different specifities are required to degrade native (intracellular) and denaturated (extracellular) PHAs (28, 52). Intracellular PHAs are amorphous and covered by a surface layer of proteins and phospholipids. On the contrary, extracellular PHAs are crystalline. PHAs are degraded intracellularly by microorganisms in the absence of a carbon source that can be used for biomass production (53, 54). Extracellular PHA depolymerases are secreted by microorganisms to hydrolyze polyhydroxyalkanoates released into the environment after the accumulating organisms have died. Their specificities, however, are not only determined by where they act. The physico-chemical properties of the polymer are also very important as described by Jendrossek *et al.* who divided the most important factors into four groups (22):

1. Stereospecificity;
2. Crystallinity;
3. Molecular mass (the lower the molecular mass the faster the degradation) and;
4. Monomeric composition of the PHA.

The mechanism of PHA hydrolysis and the important characteristics of PHA depolymerases and their regulation have been described (22). Information on the technical aspects and biodegradability of PHAs can be obtained from (55-58).

3.3. Thermo-mechanical properties

The thermo-mechanical properties of polyhydroxyalkanoates depend significantly on the chemical structure, the co-monomeric composition, the co-monomer-unit compositional distribution, the molecular weight distribution, and the average molecular weight (59-61). The homopolymer PHB is a relatively stiff and brittle crystalline thermoplastic due to the extreme stereo-regularity of the perfectly isotactic chain configuration (27, 62, 63). PHB exhibits a high melting temperature (T_m), near to its thermal decomposition temperature, and therefore leads to restricted processing possibilities. The tensile strengths of polyhydroxybutyrate and isotactic polypropylene are similar (64). However, the high crystallinity of PHB significantly limits its application possibilities, especially for biomedical applications since enzymatic attack is difficult, resulting in low degradation rates (65-69).

The incorporation of monomers other than 3HB generally leads to decreasing T_m, glass transition temperature (T_g), tensile strength and crystallinity, but to increasing elongation at break. Tensile strength describes the pull stress (force per area) required to break the material. The glass transition temperature is the temperature below which a polymer is very rigid and brittle, due to little mobility of the molecules. Plastic deformation can occur above T_g. Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) was discovered to have advanced properties compared to PHB, however PHBV still has a high degree of crystallinity (60-80%) (70, 71), which is one of the parameters when characterizing semicrystalline polymers (63). This is due to the isodimorphism phenomenon that occurs in copolymers of PHBV: 3HB units surround 3HV monomers (and vice versa for 3HV above 40 mol%) without disruption of crystallinity (64, 71, 72). Moreover, the portion of 3HV units has to be relatively high to significantly change the thermo-mechanical properties of PHBV (28). The solution seems to be the incorporation of monomeric units with longer side chains to form mcl-PHAs. Table 1 summarizes the influence of the monomeric composition on the thermal and physical characteristics of different PHAs.

Table 1 indicates that elongation at break, tensile strength, T_m, and T_g are primarily influenced by the monomeric composition and the side chain length. With the exception of the elongation at break, these thermal and physical characteristics decrease with increasing length and abundance of the pendant groups. Thus, incorporation of

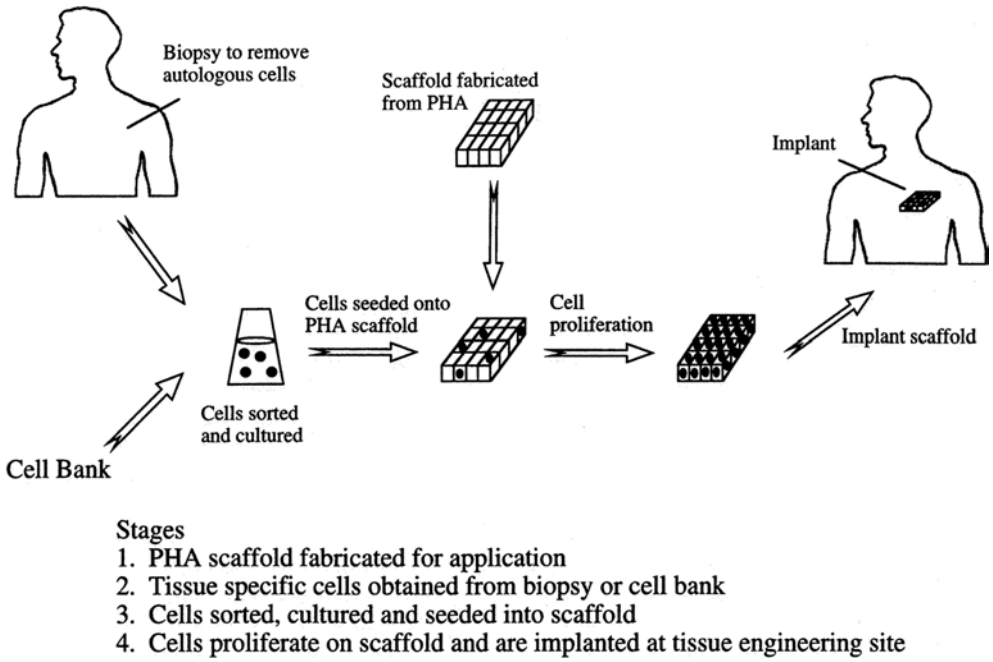

Figure 3. Principle of implanting a scaffold. The schematic diagram shows the implantation of a "mature" (i.e. three-dimensionally overgrown) scaffold. Reproduced with permission from (76).

units other than 3HB improves the thermo-mechanical properties of polyhydroxyalkanoates resulting in softer, more flexible, and less crystalline bioplastics. Poly(4-hydroxybutyrate) (P4HB) holds an exceptional position among PHAs as this homopolymer does not have a side chain and the backbone length is extended by one carbon atom compared to the 3HA backbones. Hence, this PHA is currently under investigation for biomedical applications. More detailed analysis of the thermo-mechanical properties of these polymers can be found in other recent reviews (17, 64) and references therein.

4. POLYHYDROXYALKANOATES AS TISSUE ENGINEERING MATERIALS

The material properties of polyhydroxyalkanoates qualify them for high performance biomaterials as required for directed tissue regeneration. Tissue engineering materials have to provide an interconnected porous network, which is commonly referred to as a scaffold. Like all potential scaffolding materials, PHAs have to fulfill five key requirements (76):

1. Biocompatibility.
2. Biodegradability to non-toxic products.
3. Support cell adhesion.
4. Allow ingrowth of cells.
5. Guide and organize cells within the scaffold.

Scaffolds made of polyhydroxyalkanoates are assumed to meet the first two requirements listed above. They can be prepared using most of the currently available techniques including rapid prototyping (77). The principle of preparing a PHA scaffold is illustrated in Figure 3.

4.1. Poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)

Solvent casting and particle-leaching (SCPL) techniques were applied to produce PHB foams with a controlled open-cellular structure (78). The authors of this study described a technique whereby chloroform was used as a solvent and sodium chloride as particles and proposed that their resulting three-dimensional matrix may be suitable for biomedical applications. A melt spinning technique was employed to produce PHB fibers from granulated powder (79). These fibers were spun to textile structures of various forms with the help of an embroidering technique. Previously prepared PHB films could be attached to the spun fibers to obtain implantable three-dimensional PHB patches of high mechanical strength and flexibility. Finally a low-pressure ammonia plasma treatment caused a hydrophilic surface to be produced. Comparisons between treated and untreated surfaces indicated that these modifications can lead to promoted growth of fibroblasts, making these patches highly applicable for medical purposes (79-81). Shishatskaya and Volova investigated films made from high-purity PHB and PHBV (96/4 - 70/30)[1] as matrices for *in vitro* cell culture (82). They reported PHBV to be more porous than PHB, although both exhibited otherwise identical surface properties. Cell cultures of fibroblasts, endothelium cells, and isolated hepatocytes showed high levels of attachment to the PHA films. In contrast to a preliminary study (83), not only PHB but also PHBV were found to be non-toxic. It was concluded that the purity level of the biomaterial strongly determined the biological response in addition to chemical composition and fabrication method (82).

Composite materials consisting of hydroxyapatite (HAP) and PHB or hydroxyapatite and PHBV have been shown to be sustainable scaffolds for bone tissue engineering. Ni and Wang incorporated HAP into PHB by compounding, milling, and compression molding (84). The resulting PHB/HAP composite was tested *in vitro* for the replacement of hard tissue. A layer of apatite, the major component of bones and tooth enamel, was formed on the composite surface after a short time period. Measurements of the storage modulus of the biomaterial indicated degradation of the material over time. The rate of formation and growth of the apatite layer as well as the mechanical properties could be varied by changing the HAP portion of the composite. Luklinska and Schluckwerder studied the potential of PHB and PHBV, which were reinforced by HAP, by implanting scaffolds into the tibias of rats (85). They reported lamellar bone formation that replaced the composite matrix over the 6-month implantation period and suggested that these biomaterials could be suitable candidates for bone regeneration. Koese *et al.* investigated the potential of PHB and PHBV scaffolds *in vitro* for the formation of bone tissue and reported augmented osteoblast cell proliferation and differentiation on both matrices (86, 87).

Investigations on the potential of PHB to fill nerve gaps were carried out (88). In this study conduits were prepared by rolling compressed PHB fibers in the form of rectangular sheets around an intravenous cannula. These conduits were then filled with an alginate hydrogel containing Schwann cells (SC), which surround axons of the peripheral nerve system with myelin and secrete regulatory factors. The resulting composite was implanted into rats to bridge a 10 mm gap in the sciatic nerve. In comparative tests, allogeneic SC exhibited similar matrix ingrowth and axal regeneration compared to autologous SC without deleterious immune response at the beginning. However, the extrinsic Schwann cells were rejected after six weeks as indicated by the presence of a large number of lymphocytes. The authors proposed allogeneic SC to be a good choice for nerve repair due to their immediate availability in case of an emergency. However, more research is required to prevent the observed immune response from occurring (88). In a subsequent study, the glial growth factors (GGF) were added to the alginate-PHB matrix (89). GGF are SC-specific trophic factors, which are essential for axonal regeneration following injury. The conduits were evaluated for long gap repair of peroneal nerves in New Zealand white rabbits that help to activate muscles that are responsible to lift the foot. Pure alginate-PHB and empty PHB conduits were used as controls. It was concluded that the addition of GGF greatly enhanced axonal regeneration in a time period of 120 days, resulting in less muscle mass loss compared to the controls (89). Furthermore, the application of an alginate-PHB matrix for nerve repair was successfully tested to treat spinal cord injury in rats (90).

The application of the composite PHB/triethylcitrate as a stent, in cases where life-long persistence of the stent is not necessary, failed due to inflammatory reaction to the implanted scaffold (91). Triethylcitrate was used to enhance flexibility and reduce brittleness of the biomaterial. The composite stents were implanted into the iliac arteries of New Zealand white rabbits. The in-stent lumen diameter was highly decreased due to accumulation of collagen and monocytes as well as to intercellular matrix production. Complete occlusion occurred in one case. The authors of this study speculated that PHB degradation was responsible for the vascular clotting without offering a coherent explanation for the linkage between degradation and the observed inflammatory reaction. They proposed that the portion of biodegradable material should be reduced and that the mechanical properties should be improved (91). A new scaffolding technique for PHB was developed by preparing highly porous scaffolds with an emulsion template method (92). Scaffolds were laminated to a three-dimensional matrix. The construct exhibited porosity above 90% with a median pore size ranging from 5 to 30 microns that had good interconnectivity. The researchers reported an accelerated degradation rate *in vitro* due to the porous configuration. Moreover, they proposed that the wide distribution of the pore size could favorably promote cell growth and mass transfer.

4.2. Poly(4-hydroxybutyrate) and poly(3-hydroxyoctanoate) with copolymers

Scaffolds for the regeneration of heart valves were created based on decellularized porcine aortic valves that were enzymatically degraded to obtain the extracellular matrix without changing the biochemical properties (93). The extracellular matrix (ECM) was dissolved in ethanol and then immersed in a 1% PHA/chloroform (v/v) solution followed by wet solvent elimination in a phosphate buffer solution (PBS). The resulting biomatrix/PHA hybrid was tested for biocompatibility and fluid dynamics *in vitro* as well as in rabbits and sheep. The PHAs used for the polymer impregnation were PHB, P4HB, and poly(3-hydroxbutyrate-*co*-4-hydroxybutyrate) (PHB4B). They were used to stabilize the matrix since enzymatically decellularized heart valves, which are basically a fibrous network of collagen and elastin in addition to proteoglycans, exhibit weak mechanical properties. The biomatrix/PHA hybrids showed similar fluid dynamic and morphometric characteristics compared to native heart valves. Cell culture tests showed that a mixed population of human endothelial cells and myofibroblasts attached and proliferated on all three composites, whereas mouse fibroblasts grew on P4HB and PHB4B only. Preliminary *in vivo* tests in rabbits showed PHB-treated matrices to be more biocompatible than P4HB-impregnated heart valves. However, since PHB is rather brittle and stiff, PHB4B (82/18) was chosen to stabilize the decellularized porcine aortic valves for implantation in four different sheep, of which one died during surgery. The remaining three animals were in good condition after three months. The implanted heart valve scaffolds did not exhibit any dysfunction. Similar experiments confirmed the feasibility and functionality of these hybrid composite heart valve scaffolds (94).

Rapid prototyping techniques were performed with different PHAs and co-grafted polymers to fabricate

heart valve scaffolds (95, 96). In preliminary studies, a thermal processing technique was applied to an aluminum template to produce porous P4HB, poly(3-hydroxyoctanoate) $(PHO)^2$, and poly(3-hydroxyoctanoate-*co*-3-hydroxyhexanoate) (PHOHx) scaffolds. These proved to be advantageous over PGA for the preparation and implantation of heart valve scaffolds after performing tests in pulsatile flow bioreactors (97, 99). To improve the scaffolding process, the two PHAs were used to fabricate scaffolds with the help of rapid prototyping. Human heart valves from organ donors were scanned with computed tomography (CT). Based on the acquired image, stereolithography was used for the three-dimensional reconstruction of the shape of the valve and vessel. The obtained stereolithographic plastic model was then wrapped by SCPL-treated PHA (P4HB and PHOHx) films. With the help of the thermal processing technique, a trileaflet heart valve scaffold was formed. The constructed porous 3D matrix exhibited the anatomic shape of the original heart valve and showed the desired valve function in a pulsatile flow bioreactor (95). The same technique was used to engineer a vascular stent except that magnetic resonance imaging (MRI) was employed instead of CT for the image acquisition of the aortic arch. The patient was a 12 year old, who suffered from aortic coarctation (narrowing of the aorta). The plastic model was wrapped tightly by P4HB-coated PGA patches to create an anatomically exact copy (96). Further tests were not performed but earlier *in vitro* studies showed that vascular cells attached to and migrated into scaffolds made of PHO and P4HB/PGA (100, 101). In *in vivo* investigations, autologous vascular cells from carotid arteries were seeded onto PHO heart valve scaffolds for four days and incubated for 10 more days before implantation into the pulmonary position in lambs. The animals were killed after 17 weeks. During this time period, the tissue engineered heart valves exhibited proper function. The scaffolds were covered with fibrous tissue without any thrombus formation. Similar mechanical properties of the matrix covered by neotissue were observed compared to the mechanical properties of a native heart valve (98). Only a 30% molecular weight loss of the P4HB scaffold had occurred during the 17 weeks. Nevertheless, the relatively inelastic PHO scaffold (compared to native heart valve tissue) changed its mechanical characteristics suggesting that the regenerated tissue had overtaken the heart valve function at the time of death. In a recent study, human cardiovascular patches were fabricated *in vitro*. Using the previously described bioreactor set-up, human vascular cells attached and grew into porous P4HB scaffolds. With a novel cell seeding technique, it was possible to seed both sides of the patch and to provide an optimal amount of cell culture medium on both sides by positioning the patch between two metal rings (102).

4.3. Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate)

The microbiology research group at Tsinghua University (Beijing, China), headed by Guoqiang Chen, has intensively characterized PHAs as tissue engineering materials, focusing on poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate). PHBHx showed the best performance when compared to PHB and PLA for rabbit bone marrow cell attachment and proliferation (103). The osteoblasts exhibited their characteristic morphology and, after 10 days of incubation, the cell population of PHBHx scaffolds was larger by approximately 40% compared to PHB and by 60% compared to PLA scaffolds. Wang *et al.* proposed the use of PHBHx due to its surface properties, which they described as being rougher than the ones characteristic of PHB and PLA. Hydroxyapatite was blended into PHBHx scaffolds with the aim of strengthening its mechanical properties. However, this was not successful. Furthermore, the osteoblast response was only enhanced for PHB/HAP composites due to better compatibility between PHB and HAP. Incorporating HAP into the less crystalline PHBHx matrix led to surface smoothing, which weakened the cell adhesion process (104). In another study, PHBHx was blended with gelatin. These composite films showed greater compatibility to mouse osteoblasts compared to PHBHx, again, supposedly due to increased surface roughness. Blending gelatin with PHBHx also resulted in accelerated degradation. It was proposed that increased surface porosity and decreased film crystallinity were responsible for this effect. A gelatin portion of 10% was found to be best for biodegradation, biocompatibility, and mechanical properties of the composite (105). Yang *et al.* evaluated the potential of PHBHx films to promote bone marrow stromal cell differentiation into osteoblasts. The seeded cells showed attachment, proliferation, and differentiation so it was concluded that PHBHx may be considered as a biopolymer for bone tissue formation (106). The optimal monomeric composition of PHBHx matrices for different cell types was determined by preparing PHA films with 5, 12, and 20% 3HHx content in comparison to PHB and PLA films. In general, PHB and PHBHx showed better biocompatibility compared to PLA. The osteoblast proliferation was best with PHBHx (88/12), while fibroblasts preferred PHBHx (80/20). Since the surface morphology and hydrophilicity as well as the copolymer could be varied with changing the 3HHx content, it was concluded that this PHA can be optimized according to the cell type (107).

Scaffolds based on poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) and composites are also interesting for the regeneration of cartilage tissue. A series of blended PHBHx/PHB scaffolds was fabricated with SCPL technique (108). The PHBHx-to-PHB ratios were 1:0, 2:1, 1:1, 1:2, and 0:1. Chondrocytes from rabbit articular cartilage were seeded onto the prepared matrices and investigated for adhesion and proliferation over 28 days. In all cases, the cells attached to the scaffolds and resembled to fibroblasts during the second half of incubation. Ingrown chondrocytes were predominantly spherically shaped and more numerous within the 1:2 and 2:1 PHBHx/PHB scaffolds. The authors of this study explained the superior biocompatibility of these composites on the basis of improved surface properties. They proposed PHBHx/PHB blends consisting of crystalline and amorphous domains, in which PHB acts as physical crosslinker and filler. For this reason, the blended scaffolds were described to be rigid but not brittle. However, no explanation was provided as to why the 1:1 PHBHx/PHB matrix did not perform as well as the other blends in terms of chondrocyte proliferation. In

addition, the monomeric distribution of the PHBHx used was not stated. In a subsequent study, ECM production of articular cartilage chondrocytes grown on the same series of PHBHx/PHB blends was investigated (109). This time, the 2:1 composite was the most capable of providing attachment and ingrowth of collagen type II produced by the chondrocytes, which is characteristic of the extracellular matrix. Similarly, PHBHx/PHB blends were shown to have better surface properties than pure PHA scaffolds (110). The crystallization behavior of these composites and their interactions with rabbit chondrocytes were studied (111). The PHBHx/PHB ratios were 1:0, 3:1, 1:1, 1:3, and 0:1 with 12 mol% 3HHx portion within the PHBHx copolymer. In comparison to the investigations previously described, films were used instead of SCPL-prepared scaffolds. The 1:1 blend was shown to be the optimal material for chondrocyte adhesion. The authors stated that the crystallinity of the blend increased with increasing PHB content and that the degree of polymerization significantly influenced surface chemistry, polarity, and free energy. Surface polarity increased as crystallization was reduced, due to a larger amount of oxidized carbon atoms on the surface. The surface free energy was highest for the blend with equal PHB and PHBHx content and stated as reason for enhanced chondrocyte adhesion. An increase in elongation at break and a decrease in tensile strength with increasing PHBHx (87/13) content were reported for PHBHx/PHB blends (112).

Many more studies on PHAs as potential tissue engineering materials have been performed and the number is increasing. More detailed information can be obtained from (9, 10, 38, 69, 74, 76, 113-116).

5. FUNCTIONALIZED POLYHYDROXYALKANOATES

Reducing costs is an important priority for the mass production of polyhydroxyalkanoates to replace conventional plastics. However, further improvements to products are also necessary to develop the required properties for specialized applications, for example in tissue engineering as described above. Most common PHAs like PHB and PHBV are inert due to the alkylic nature of their side chains. However, it is desirable to have functionalized polyhydroxyalkanoates in order to tailor their thermo-mechanical properties or to make them amenable to chemical modification. The latter can be used to graft other polymers onto the PHAs or to modify the surface properties.

5.1. Discovery

Although Davis reported on the discovery of poly(3-hydroxybutyrate-*co*-3-hydroxybut-2-enoate) from *Nocardia* sp. grown on n-butane in 1964 (117), the work of Lageveen *et al.* can be seen as the first tailored biosynthesis of PHAs with side chains other than alkyl groups (118). In their experiments, *Pseudomonas putida* GPo1 (commonly known as *Pseudomonas oleovorans* ATCC 29347) grew on C6 to C12 n-alkanes and 1-alkenes and produced either medium-chain-length poly(3-hydroxyalkanoates) or poly(3-hydroxyalkanoates-*co*-3-hydroxyalkenoates) corresponding to the substrates fed under nitrogen deficiency conditions. The degradation of fatty acids via the beta-oxidation pathway and the polymerization of the degradation products to PHAs is characteristic of pseudomonads belonging to the rRNA homology group 1 (14, 16). It was reported that the copolymers produced always consisted of monomers with the same length as the fed substrate and of monomers shorter by one or two C2 units. It was concluded that the monomeric composition of the PHAs should be a reflection of the substrates supplied taking into consideration a partial C2 loss during polymerization. The same phenomena were observed when 1-alkenes were fed. However, the incorporation of these olefinic molecules appeared to be less efficient since no pure poly(3-hydroxyalkenoates) but poly(3-hydroxyalkanoates-*co*-3-hydroxyalkenoates) were obtained. Lageveen and co-workers deduced that 1-alkenes were oxidized on both ends resulting in alkanoic and alkenoic acids. These results indicated the potential of pseudomonads for the tailor-made manufacturing of PHAs with desirable properties.

5.2. Biosynthesis and production

Only limited studies involving the accumulation of functionalized PHAs in bacteria other than *Pseudomonas* species have been reported. Polyhydroxyalkenoates were found in *Chromobacterium* sp. (119-121), *Rhodospirillum rubrum* (122, 123), and *Burkholderia* sp. DSMZ 9243 (124, 125). It should be noted that this does not mean that other bacteria are not able to produce functionalized PHAs. However, since pseudomonads generate tailored PHAs at a high rate of productivity, many researchers have focused on investigating the pathways and PHA production possibilities of *Pseudomonas* species rather than looking for new potential microorganisms for the formation of functionalized PHAs. Three pathways are involved in the formation of mcl-PHAs (see Figure 4) (21, 126).

Pseudomonads belonging to rRNA homology group 1 are able to metabolize alkanes, alkanols, alkanoates, and their olefinic analogs, respectively (118). PHAs are formed via the fatty acid beta-oxidation cycle. *P. oleovorans* is the most common representative of this pathway in addition to other fluorescent species (127). Except for *P. oleovorans*, all pseudomonads are also capable of producing mcl-PHAs from unrelated carbon sources like sugars and other carbohydrates (64, 128). Metabolism of unrelated substrates occurs either via chain elongation or *de novo* fatty acid synthesis. The last pathway is characteristic for *P. putida*. More detailed information on the biosynthesis of PHAs in pseudomonads can be obtained from (14, 16, 21, 64, 128, 129).

Reviewing the past literature on *Pseudomonas* species is somewhat confusing in terms of the terminology used. In 2001, *Pseudomonas oleovorans* ATCC 29347, by far the most studied species for mcl-PHA production and a characteristic example for the fatty acid beta-oxidation pathway, was reclassified as *Pseudomonas putida* GPo1. *P. putida* strains form a second group of characterized species, which are representative of the *de novo* fatty acid synthesis pathway (130). Van Beilen *et al.* reported that the 16S

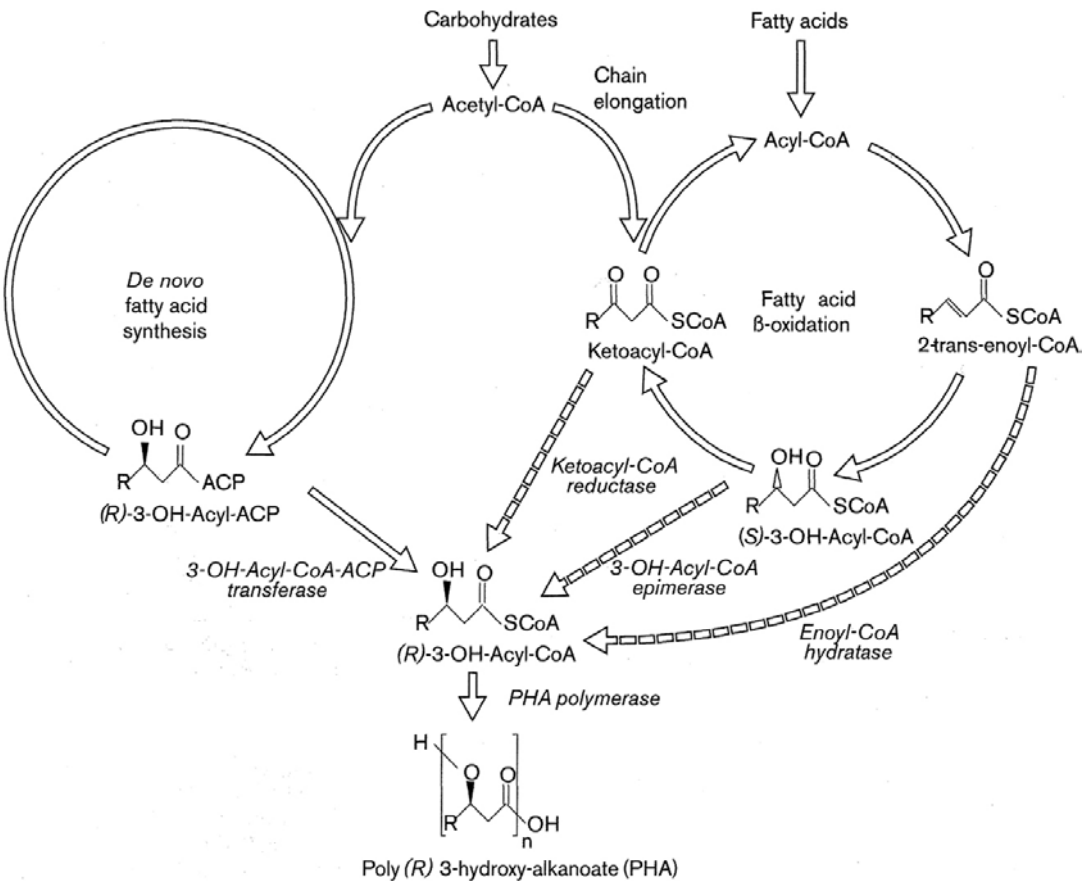

Figure 4. Major pathways of mcl-PHA synthesis in pseudomonads. Reproduced with permission from (21).

rRNA sequences of *P. oleovorans* ATCC 29347 was identical to that of *P. putida* ATCC 17633, which had been deposited earlier (130). Therefore, *P. oleovorans* ATCC 29347 and *P. putida* GPo1 are synonymous.

PHAs with functional groups, predominantly in the form of terminal double bonds within the side chains, have also been discovered in *Pseudomonas* sp. A33 (131, 132), *Pseudomonas cichorii* YN2 (133), *Pseudomonas aeruginosa* NCIB 40045 (134), 44T1 (135) and PR3 (136), *Pseudomonas corrugata* (137), as well as in *Pseudomonas citronellolis* ATCC 13674 (138). To date, microbiologically produced polyhydroxyalkanoate copolymers with the following side chain functionalities have been reported: alkylbranched (139, 140), cycloalkyl (141), ester (142), aromatic (143-151), phenoxy (152-154), cyano (155, 156), nitro (155, 157), fluorine (158-160), chlorine (161), bromine (156, 162, 163), alkine (164), oxo (165), acetoxy (142, 165), alkoxy (166), and epoxy (133, 135, 167). However, the majority of these functionalized PHAs have only been generated in analytical amounts.

Apart from the pioneer work (118), most recent publications dealing with functionalizing PHAs based on terminal double bonds in the side chains can be traced back to the work of Fritzsche *et al.* (168), Kim *et al.* (169), and Bear *et al.* (167). *P. putida* GPo1 or similar *P. oleovorans* strains were used to produce poly(3-hydroxyoctanoate-*co*-3-hydroxyundec-10-enoate)s (PHOUs) (75, 170-181). PHOUs also include monomer units other than 3HU(=) and 3HO since the C2 reduction reported by Lageveen *et al.* occurs during PHA formation (118). The produced PHA is illustrated in Figure 5. However, the term PHOU became so established that the saturated monomeric units derived from octanoate are classified as "O" while the unsaturated monomeric units derived from 10-undecenoic acid are classified as "U". By supplying octanoate and 10-undecenoate as substrates to *Pseudomonas oleovorans* in batch mode, the degree of unsaturation was controlled from nearly 100 to 0% within the polymer while slightly increasing cell yield, PHOU yield, and PHOU content (75).

Limited studies have been performed to optimize the production process of functionalized PHAs. Batch and chemostat strategies were compared for the production of PHOUs (174). As in earlier mcl-PHA production studies with *P. putida* (182, 183) and in PHBV production with *Ralstonia eutropha* (184), continuous culture was proposed to be the most suitable method for the production of PHOUs (174). Cell yield in the chemostat experiments was slightly lower but, more importantly, continuous culture resulted in exactly defined monomeric compositions at steady state compared to batch culture, in which the PHA production depended on the growth stage of the cells.

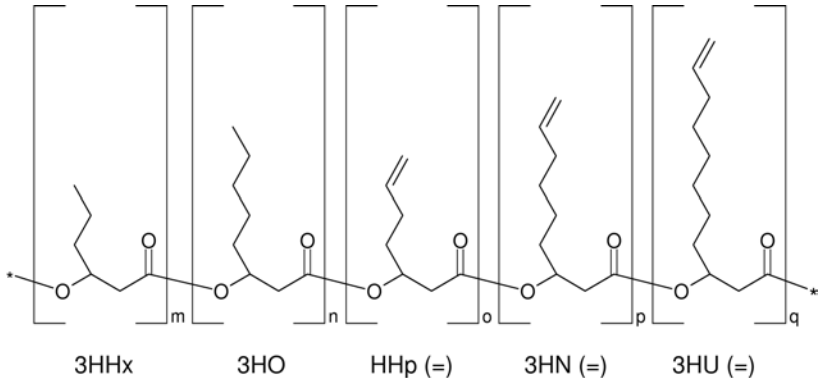

Figure 5. Structural formula of PHOU. 3HHx: 3-hydroxyhexanoate; 3HO: 3-hydroxyoctanoate; 3HHp(=): 3-hydroxyhept-6-enoate; 3HN(=): 3-hydroxynon-8-enonate; 3HU(=): 3-hydroxyundec-10-enoate. Kim *et al.* also reported saturated C5, C7, C9, C10, and C11 units to be present. These are not included in the structural formula since they were only detected in trace amounts (169).

Hartmann *et al.* reported T_m and T_g of PHOUs to decrease with increasing unsaturated monomer content (174), confirming the findings of Park (75). The glass transition temperature decreased from –33.1 degrees Celsius in PHO[2] to -49.3 degrees Celsius with purely unsaturated PHA derived from 10-undecenoate. The melting temperature of PHO was 58.1 compared to 39.9 degrees Celsius for PHOU (47/53). PHOUs with an unsaturated portion higher than 53% were completely amorphous. It has yet to be demonstrated whether this phenomenon occurs because of the increase in the average side chain length with increasing "U" fraction or whether it is due to the terminal double bonds. The effect of side chain elongation is known. Moreover, the double bonds had a distinct impact on the melting temperature without significantly affecting the glass transition temperature after comparing poly(3-hydroxypent-4-enoate) homopolyester produced by *Burkholderia* sp. to the fully saturated analogue poly(3-hydroxvalerate) (125). This might explain the stronger temperature drop in T_m in comparison to T_g observed by Hartmann *et al.* (174). The same research group could vary the thermal properties by introducing 5-phenylvalerate into PHOU (147).

Sun *et al.* performed a series of studies to optimize the production of mcl-PHAs by feeding alkanoic acids and glucose to *Pseudomonas putida* KT2440 (185-189). In contrast to the most common method of producing high yields of PHAs by employing a two-stage fed-batch fermentation with nitrogen or phosphorous limitation during the second stage, they found that carbon-limited, exponential feeding of nonanoic acid yielded in the best production of mcl-PHAs (187). Furthermore, the PHA yield was increased by 30% when glucose was co-fed (186). The principle of exponential carbon feeding was then applied to obtain functionalized PHAs bearing terminal double bonds by co-feeding nonanoic and 10-undecenoic acid (188).

PHAs with non-terminal double bonds were produced by growing *Pseudomonas putida* IPT046 on carbohydrates (190). As stated above, the incorporation of functional groups usually occurs from related substrates that are oxidized via the beta-oxidation pathway and not via *de novo* fatty acid synthesis from carbohydrates.

5.3. Chemical synthesis

Only stereo specific polyhydroxyalkanoates in R configuration are 100% biodegradable (191). This characteristic hampers the chemical synthesis of fully biodegradable PHAs. The chemical production of PHAs generally occurs from the ring-opening polymerization of beta-lactones. Numerous attempts have been made to form optically pure PHAs using different catalysts that result in highly but not purely isotactic polyhydroxyalkanoates (60, 192, 193). Recently, Kramer and Coates polymerized fluorinated beta-lactones to PHAs with halogenic functionalities (194). According to their C-NMR analysis, these functionalized PHAs were perfectly isotactic. They also copolymerized fluorinated beta-lactones with beta-butyrolactone to obtain functionalized copolymers. PHAs with amine function were produced by developing a method to chemically synthesize PHAs with carboxylic or primary amine pendant groups (195).

6. MODIFICATIONS FOR PROPERTY IMPROVEMENTS

For biomedical and tissue engineering applications a single type of polymer or copolymer is often insufficient to meet the needs of complex systems. The introduction of functionalities to PHAs is the first step in the process of converting them into candidates for specific applications. Consequently they can be further improved by modifying their chemical and physical properties.

6.1. Chemical modifications

The majority of functionalities can only be introduced to PHAs in analytical amounts due to the toxicity of the substrates. The resulting low productivities and yields make mass production impractical. While unique properties as a result of functional groups can increase the value of products, it is likely that this will be achieved by chemical modification of PHAs produced via fermentation.

The principles of chemical modifications of PHAs and their results are presented here. Details regarding the chemistries behind these modifications can be obtained from (196) and (197) and references therein.

6.1.1. Chlorination

A good example for chemical modifications is the introduction of chlorine within PHA side chains. Doi and Abe obtained copolymers with 3-hydroxy-omega-chloroalkanoate monomers after co-feeding 1-chlorooctane (161). Arkin and co-workers used cheaper carbon sources to produce unsaturated PHAs and chemically chlorinated the obtained copolymers, leading to higher chlorination yields compared to Doi and Abe (170, 198). However, this chemical modification also led to hydrolysis of the PHAs and therefore molecular weight loss, which is a common problem encountered during chemical modification. Moreover, as in this example, modification by chemical means is often undesirable as hazardous reaction conditions may occur. While scl-PHAs are often too hard and brittle, some mcl-PHAs may be too soft and sticky for fine applications, especially if produced from certain inexpensive substrates. The substitution of terminal double bonds with chlorine led to an increase in melting temperature, glass transition temperature, and crystallinity (198). These functionalized PHAs were used as templates to obtain their corresponding sodium sulfate salts and quaternary ammonium salts (170). Moreover, the chlorine could be substituted with benzene to form copolymers with side chains bearing phenyl groups. This substitution reaction also led to crosslinking between polymeric side chains.

6.1.2. Carboxylation

Advantageous functionalities cannot always be introduced by biosynthesis. Polyhydroxyalkanoates bearing pendant carboxyl groups in their side chains are desirable polyesters since these functional groups are reactive and can easily be used as templates for grafting other molecules onto the initial polymer, e.g., bioactive molecules, peptides, and hydrophilic oligomers and polymers. Due to enhanced hydrophilicity, PHAs bearing pendant carboxyl groups are promising candidates for tissue engineering and biomedical applications such as *in vivo* drug delivery systems (197, 199, 200). Biosynthesis of this kind of PHA has not been successful to date, with the exception of poly(beta-malic acid) (20), due to toxicity of the di-carboxylic acids supplied as substrates (201, 202). Although it may be possible to produce PHAs with ester groups, which can be subsequently transformed into carboxyl groups by esterases, an alternative means of obtaining this kind of polyester using artificial carboxylation has been reported (171, 175, 181). The problem of high molecular weight loss during the carboxylation reaction was overcome (181). A further issue is the poor mechanical properties exhibited by carboxylated PHAs (197). To date only PHOUs have been used to produce PHAs with carboxyl functionality. The usefulness of terminal carboxyl groups as reactive intermediates was demonstrated by transforming them into their amide and ester derivatives (203). PHOU was first coupled with 11-mercaptoundecanoic acid via radical addition to obtain side chains with thioether bonds and terminal carboxyl groups. These carboxyl groups were then transformed into PHOU amide and ester derivatives using tridecylamine or octadecanol, respectively. The resulting functionalized PHAs did not suffer from molecular weight loss and showed a very high degree of crystallinity as compared to PHOU. Polyhydroxyalkanoates with reactive carboxylic side groups were also studied as starting material for the preparation of co-grafted polymers (see below).

6.1.3. Hydroxylation

Several studies have been performed aimed at enhancing the hydrophilicity of PHAs by hydroxylation (176, 204, 205). Lee *et al*. hydroxylated up to 60% of the unsaturated side chains of PHOUs (45% - 93% degree of unsaturation) using basic $KMnO_4$ without significant loss of molecular weight (176). In contrast, complete conversion of double bonds into hydroxyl groups was achieved by applying the principle of hydroboration-oxidation to PHOUs (25% unsaturated side chains), albeit at the expense of the molecular weight (205). Due to increased hydrophilicity, the hydroxylated copolymers were soluble in polar solvents such as ethanol and methanol. The hydroxylation of pure PHUs by hydroboration-oxidation led to a conversion yield of nearly 100% (204). The resulting copolymers were highly reduced in molecular weight, however their hydrophilicity was increased to the extent that they were partially soluble in water. Independent of the chemical route applied, with increasing conversion yield molecular weight was observed to decrease. Consequently there appears to be a trade-off between hydrophilicity and mechanical properties. Hydroxyl functionalities can be useful following further chemical modifications (206). Double bonds were transformed into thioether bonds via the radical addition of 11-mercaptoundecan-1-ol. The resulting derivatives with hydroxy functionality [A] were then esterified with cinnamic acid to obtain aromatic functionality [B], sulfatized with $ClSO_3H$ [C], or modified with tert-butyldimethylsilyl-protected coumaric acid [D]. The latter derivative exhibited a phenoxy group, which was furthermore sulfatized with $ClSO_3H$ to form zosteric acid-labeled PHOU derivatives [E]. In contrast to the other functionalized polymers and PHOU, derivatives [C] and E] were soluble in methanol and dimethyl sulfoxide (DMSO) owing to the sulfuric function. The coupling of the hydroxyl group of [A] with tert-butyldimethylsilyl-protected coumaric acid and the subsequent sulfatization resulted in a severe molecular weight loss. Zosteric acid-labeled PHAs were proposed as coatings to protect surfaces from biofouling (207).

6.1.4. Epoxidation

Epoxy groups are of high interest because they are highly reactive under mild reaction conditions and can, therefore, be easily transformed into polar and ionic groups (197). Similarly to the chemical modifications described above, PHAs bearing terminal double bonds can be used as original polymers to carry out epoxidation reactions. After the successful epoxidation of poly(beta-malic acid) derivatives containing lateral double bonds (208), a series of PHOUs and poly(3-hydroxybutyrate-*co*-3-

hydroxyvalerate-*co*-3-hydroxypent-4-enoate) were deoxidized with m-chloroperbenzoic acid (MCPBA) at room temperature in CH_2Cl_2 (167). The conversion yield was reported to be 100% and no molecular weight loss of the polymeric chain occurred due to the mild reaction conditions. Epoxidized PHAs were also produced by growing *Pseudomonas putida* GPo1 on different ratios of octanoic acid and 10-epoxyundecanoic acid. However the cell and PHA yields were low compared to the production of PHOU, indicating that production of polyhydroxyalkenoates followed by mild chemistry may be more appropriate in order to attain desired functionalities. Park and co-workers performed several studies to evaluate the production of epoxidized PHAs, their properties, and their potential for further modifications by crosslinking (178, 209, 210). Using the same reaction conditions as described previously, partially epoxidized PHOUs were tested for their thermal properties. Melting temperature (T_m) decreased while glass transition temperature (T_g) increased with increasing epoxidation yield. Valentin *et al.* oxidized 45% of the double bonds of nearly pure poly(3-hydroxypent-4-enoate) to epoxy groups (125). They reported the epoxidation with MCPBA to be stereochemically unspecific and confirmed the observation of a higher glass transition temperature as result, owing to increased intramolecular interactions.

6.2. Curing

As mentioned above, many functionalized PHAs often exhibit poor mechanical properties. Curing describes the toughening and hardening of polymers by crosslinking the polymeric side chains. Crosslinking has been studied intensively to overcome the main weakness of mcl-PHAs. Crosslinked side chains can be induced naturally, chemically, and by radiation.

6.2.1. Natural crosslinking

Even though double bonds are not as reactive as other functional groups, they are vulnerable to oxidation under ambient conditions. This process is called autoxidation. Schmid *et al.* studied the autoxidative behavior of mcl-PHAs (211). They produced polymer films comprised of PHOUs with varying degrees of unsaturation by solvent casting. These PHOU films of 500 microns thickness were then stored at 60 degrees Celsius in the presence of air. The degree of autoxidation was monitored over a time period of 3 months. It was found out that PHOU alteration was highly dependent on the amount of double bonds. While PHOUs comprising less than 10% double bonds became degraded over time, the molecular weight of PHOUs with a high content of unsaturation (above 50%) started to increase after a short time period. This increase suggests that autoxidation may occur first, followed by crosslinking of the side chains. The authors concluded that PHAs with a double bond content of more than 50% should be stored below -5 degrees Celsius in an inert gas to preserve the polymeric structure. The shelf-life of PHUs (100% unsaturation) was calculated to be 8 months under ambient conditions before the entire polymer is crosslinked to a macroscopic network. The complete crosslinking is called gelation.

It is not surprising that natural crosslinking of functionalized PHAs occurs even faster if the polymeric side chains contain epoxy groups. The crosslinking upon exposure to air of unsaturated PHAs produced from linseed oil (PHA-L) was compared with their epoxidized derivatives (212). The unsaturated PHA-Ls were comprised of even-numbered monomeric units up to C14, of which 51% contained lateral or terminal double bonds. Oxidation with MCPBA resulted in a 37% epoxidation yield. Natural crosslinking was accelerated as epoxidized PHA-L films started to crosslink in less than 25 days while autoxidized PHA-Ls began to form a network between 50 and 75 days. In general, crosslinked PHA-Ls showed improved mechanical properties as seen by increases in tensile strengths and Young's moduli. However, fully crosslinked PHA-L films originating from autoxidized PHA-Ls were described to be glassier than elastomeric. The fact that the mechanical properties of both crosslinked PHA-L types differed was explained by a difference in the crosslinking mechanism.

6.2.2. Chemical crosslinking

The process of crosslinking can be expedited using various chemical agents. Several peroxides with and without multifunctional co-agents were tested for their potential to crosslink fully saturated poly(3-hydroxyoctanoate-*co*-3-hydroxyhexanoate-*co*-3-hydroxydecanoate) (PHOHxD) and PHOUs (172). The peroxides were classified as vinyl and non-vinyl specific. As expected, the presence of double bonds improved the crosslinking of the polymeric side chains independently of the peroxide used. The non-vinyl specific peroxides, lauroyl and benzoyl peroxide, favorably catalyzed the crosslinking reaction with the help of ethylene glycol dimethacrylate and triallyl cyanurate as co-agents. In contrast, the crosslinking reaction was reduced when these co-agents were added to the vinyl specific peroxides dicumyl peroxide and 2,5-dimethyl-2,5-di(t-butyl peroxy) hexane (DBPH). The change in material properties was described to be positive regarding the reduction or elimination of crystallinity and improved elastic response defined as tensile set. However, both crosslinked PHOHxD and PHOUs exhibited a loss in tensile strength and tear resistance and in decreased tensile modulus. Gagnon *et al.* concluded that the molecular weights of the PHAs used were too low with below 200 000 g/mol and proposed that molecular weights of between 200 000 to 500 000 g/mol should be more appropriate for peroxide crosslinking. The same research group applied sulfur vulcanization to different PHOUs to overcome the disadvantages of peroxides as crosslinking agents (173). While sulfur vulcanization resulted in the same property improvements as caused by peroxide crosslinking, the negative impact on the mechanical properties was reported to be less severe when sulfur or dipentamethylene thiuram tetra/hexa sulfide (DPTT) was applied as vulcanization agents. Nevertheless, the resulting crosslinked PHOUs also exhibited a decrease in tensile strength, tensile modulus, and tear resistance. Hexamethylene diamine (HMDA) was employed to crosslink epoxidized PHOUs (213). Each of the amino groups in HMDA was coupled with two epoxy groups of the polymeric side chains to form tertiary amino and two secondary hydroxyl groups. With this approach a degree of almost 100% crosslinking was obtained. The crosslinking

reaction was accelerated with increasing content of epoxy groups, resulting in increased T_g and relative storage moduli. In another investigation, succinic anhydride and 2-ethyl-4-methylimidazole were used as initiator to chemically crosslink epoxidized PHOUs (209). The resulting PHA gels contained diester crosslinks and exhibited very similar property improvements (213).

6.2.3. Physical crosslinking

A major drawback of chemical stimulation of natural processes is the regular presence of environmentally hazardous agents and additives. Residual chemicals may remain attached to polymers and therefore affect biodegradability and biocompatibility. Even if they can be removed, the chemical groups providing the crosslinks may also have a negative impact on the biological properties. An alternative means of accelerating natural or chemically induced crosslinking is by applying external energy in the form of irradiation. The first report on physical crosslinking was published by de Koning *et al.* (214). They applied electron beam (EB) radiation to PHAs with 0, 4, and 15% unsaturation at different doses. While the saturated PHAs showed no gelation but only degradation of their polymeric chains, the PHAs with 4 mol% and 15 mol% double bonds in their side chains showed 88 wt% and, respectively, 93 wt% gel fractions. Crosslinking PHAs with a high content of double bonds resulted in non-crystallizable polymers. The dynamic modulus and T_g could be elevated by increasing the radiation dose from 30 to 500 kGy. These properties were attributed to higher crosslink densities resulting from increased energy exposure. The polymers were described as true rubbers with constant properties over the temperature range from T_g (less than -15 degrees Celsius) to the thermal degradation temperature (170 degrees Celsius). Moreover, the crosslinked PHAs could be degraded by *P. fluorescens* GK 13.

The potential of gamma-irradiation was investigated to crosslink PHAs produced from tallow (PHA-tal) using *P. resinovorans* NRRL B-2649 (215). Due to the high content of oleic acid in tallow, PHA-tal exhibited 11% unsaturation. The double bonds were found in C12 and C14 monomeric units of the polymer. PHA films were prepared by solvent casting. The polymeric side chains were successfully crosslinked forming partly insoluble gels in chloroform. Increasing the radiation dose from 25 to 50 kGy supported gelation as well. As opposed to reports where PHAs classified as either “crosslinkable” or “degradable” when exposed to the same conditions, Ashby *et al.* pointed out that crosslinking and degradation of the polymeric chains usually co-exist during the process of ionizing radiation. They observed a severe loss in molecular weight when comparing irradiated with untreated PHA-tal films. The gelation of PHA-tal was enhanced by adding 10% (w/w) linseed oil, which is known for its natural polymerization under ambient conditions. However, completely crosslinked films were not produced despite the high level of double bonds attributed to the presence of highly unsaturated linseed oil. Despite this effect, linseed oil reduced the rate of chain scission upon gamma-irradiation. Compared to PHA-tal, the irradiated films showed increased Young’s modulus and tensile strength, making the polymers stronger and more rigid. The thermal properties were not affected by irradiation or addition of linseed oil. Comparing the thermo-mechanical properties of treated and untreated films, it was concluded that linseed oil rather functions as a plasticizer instead of being crosslinked with the polymeric side chains. As opposed to de Koning *et al.* (214), Ashby and colleagues described their PHA-tal gels to be partly biodegradable based on growth experiments of *P. resinovorans* NRRL B-2649 on crosslinked and untreated PHA-tal films. The biodegradation of irradiated linseed oil PHA-tal films was enhanced most likely due to the parallel degradation of the linseed oil by lipases.

Poly(3-hydroxypent-4-enoate) homopolymer was exposed to UV-irradiation to form crosslinks between the polymeric side chains (125). Double bonds in Fourier transform infrared spectroscopy (FTIR) spectra were hardly detectable after treatment. However this method of optimizing the crosslinking process was not viable as increased brittleness of the treated PHA films was observed. The crosslinking behavior of PHAs produced from soybean oil (PHA-soybean) by *P. oleovorans* was subject of another study (216). PHA-soybean films with a degree of 10% unsaturation were exposed to UV-irradiation in the presence of the chemical crosslinking inducers benzophenon, benzoyl peroxide and/or ethylene glycol dimethacrylate (EGDM). With 81 to 89 wt%, the crosslinking yields were slightly lower compared to the 93 wt% crosslinking yield of PHA-soybean when no chemical inducer had been added. However, the time to reach gelation was reduced from 2.4 to 1 day in the presence of benzoyl peroxide and EGDM. Despite the positive impact of UV-irradiation on the crosslinking rate, formation of the macroscopic network was even faster (0.1 days) when PHA-soybean films were exposed to daylight at 60 degrees Celsius. The glass transition temperature of the irradiated films was increased by 15 to 30 degrees Celsius. UV-irradiation was applied to PHA films and resulted in the discovery that initiators had to be added to film casting solutions to obtain high degrees of crosslinking (217). Analysis of a number of photoinitiators revealed that diphenyl(2,4,6-trimethylbenzoylphosphane oxide) (TPO) was the most effective agent as, after 10 seconds of irradiation in the presence of 3 wt% TPO, a gel fraction of approximately 95% was obtained. While no melting endotherm was observed in the differential scanning calorimetry (DSC) chromatogram, the glass transition temperature increased from -51 to -46 degrees Celsius. Successful treatment of the irradiated PHU films with an aqueous depolymerase solution indicated they were biodegradable.

Dufresne *et al.* investigated gamma-irradiation at different doses for the crosslinking of PHOUs, particularly those with 33 mol% vinyl groups (218). Irradiation in air resulted in an increased gel fraction and molecular weight loss, which was correlated to an increasing radiation dose. The crosslinking yield could be significantly increased when the PHOU films were exposed to gamma-rays in the presence of pure nitrogen. The lowest radiation dose of 20 kGy was sufficient to obtain nearly completely insoluble

PHOU gels indicating optimal crosslinking. Swelling measurements and subsequent calculations of the molecular weight between crosslinks confirmed the advantage of nitrogen over air. Despite optimal crosslinking, Dufresne *et al.* described the polymeric gels as imperfect, elastically reversible material, because crystallinity could not be completely removed. The melt and glass transition temperatures were hardly affected by gamma-irradiation, however the irradiated samples did not degrade as much indicating a gain in stability of the material.

Bassas *et al.* compared the naturally and UV-induced crosslinking reactions of polyunsaturated (36.5%) PHA-Ls obtained from cultures of *P. aeruginosa* 42A2 (219). They used the degree of unsaturation, before and after crosslinking, to determine the level of gelation. Autoxidation and UV-irradiation resulted in a decrease of unsaturated monomers so that the formerly viscous and tacky biopolymers were described to have become smooth, less sticky, and rubber-like. Moreover, they were not soluble in organic solvents, although the melting temperature remained unchanged while T_g was slightly increased. It was concluded that natural aging (6 months) under light exposure was sufficient to convert the sticky polyunsaturated PHAs into smooth films. This process could be significantly accelerated (less than 24 hours) with UV treatment, however the radicals that were responsible for the crosslink formation may have also promoted the scission of the polymeric chains. In a subsequent study, the PHA-Ls previously described and their UV-crosslinked analogues were investigated at a microscopic level (220). Transmission electron microscopy (TEM) and atomic force microscopy (AFM) analysis showed that crosslinked PHA-Ls exhibited a reticular structure. AFM furthermore revealed that crosslinks between polymeric chains were responsible for the curing of the material.

6.3. Surface modifications

The application spectrum of natural PHAs as tissue engineering materials is very limited, owing to their hydrophobic character. Ideally, biopolymeric scaffolds for tissue regeneration should exhibit a certain degree of hydrophilicity to promote cell attachment. Moreover, as discussed above, the introduction of hydrophilic groups along the polymeric chains may weaken the bulk properties of PHAs. It may therefore be desirable to only modify the polymeric surface. As presented below, biopolyesters do not need to exhibit initial functional groups for surface treatment.

6.3.1. Plasma modification

The introduction of functional groups to PHAs can be achieved by plasma treatment. Plasma is a partially ionized gas that contains a high portion of ions and electrons serving as charge carriers. The ionization is induced by applying external energy, commonly in the form of electrostatic or electromagnetic fields, to the gas in a plasma reactor. Plasma formation is promoted by keeping the pressure low in the plasma chamber. The radical gas molecules attack any material that is also present in the plasma reactor. The resulting "damage" may subsequently induce desirable changes to the surface morphology. This technique can therefore be used to improve the surface properties of polymers including polyhydroxyalkanoates. In contrast to increasing the hydrophilicity of the entire polymer by substituting vinyl with polar groups, plasma modification or plasma polymerization can be used to introduce hydrophilic groups to the surface only. As a result, the hydrophobic core remains intact and serves as mechanical support while the surface exhibits increased wettability, subsequently becoming attractive for cell ingrowth.

The most common method to determine the wettability of a material is the measurement of the contact angle at the polymer/liquid interphase. However, the result of any static contact angle measurement does not necessarily allow for a solid conclusion about hydrophilicity/hydrophobicity and, therefore, about potential cell response and protein adsorption. This is due to the phenomenon of contact angle hysteresis (221). Liquid drops on a surface undergo a shape and volume transition without a change in interfacial area. Consequently, the contact angle changes but the wetted surface area remains the same. Thus, contact angle measurements should be performed in dynamic fashion to account for the angle hysteresis phenomenon.

Plasma polymerization was used to deposit thin fluorocarbon coatings of 100 to 400 nm thickness on PHB membranes (222). Argon acted as carrier gas for the plasma treatment of PHB with perfluorohexane (PFH) and hydrogen. The fluorocarbon coatings provided PHB with a smooth surface that did not reduce the biocompatibility properties. In addition, blood clotting tests revealed that coatings of 200 nm and more thickness increased blood clotting time due to reduced surface energy from 50 to below 15 mN/m. Mas *et al.* investigated the change in surface properties by exposing PHBV to different plasmas. Oxygen plasma was used to increase surface energy (223, 224). The formation of oxygen radicals resulted in the incorporation of oxygen in the form of carbonyl, carboxyl, and ester groups in the outer layers of the PHBV films. The total oxygen content was increased by 12-13%. As expected, the wettability decreased over time as the contact angle of water continuously increased from 40 degrees after the first day of plasma treatment to a stable 62 degrees value after 70 days. This was lower than the 70 degrees contact angle value obtained for untreated samples. Therefore, oxygen plasma treatment of PHBV films resulted in a very moderate increase in hydrophilicity given that dynamic contact angle was not measured. Chain cleavage resulting in a molecular weight loss and a decrease in T_g was also reported. An additional study was performed to compare the altered surface properties generated by plasma treating PHBV films with Ar, O_2, H_2O, H_2O /O_2, and H_2O_2 (224). With the exception of argon, a treatment of 5 min or less at either 45 or 75 W was sufficient to reduce the contact angle by 30 degrees or more. The exposure to argon (in this study used as plasma and not as a carrier gas), had to be adjusted to 30 min at 75 W to obtain a similar increase in wettability. The lowest contact angles were achieved with H_2O/O_2 ($TETA_{H2O}$ = 37 degrees) and H_2O_2 ($TETA_{H2O2}$ = 38 degrees). As observed

with oxygen, all treated samples exhibited a loss in wettability over time, without returning to their hydrophobic state before treatment. The improved wettabilities caused by the treatment with H_2O and H_2O_2 containing plasmas were explained by the likely formation of OH radicals leading to hydroxyl groups on the surface. The exposure to argon or oxygen plasma resulted predominantly in the introduction of carbonyl groups to the PHBV surfaces. A direct correlation between the power applied and the wettability obtained for PHBV films after exposure to oxygen plasma was reported (225). The more power that was applied and the longer the plasma treatment, the higher the amount of oxygen that was measured on the surface, resulting in increased hydrophilicity. The treated PHBV films did not absorb significantly more water because the plasma treatment attacked the surface of the polymers only.

The radical grafting of amide and imino groups onto PHA surfaces was demonstrated by exposing PHBV films to allylamine low-pressure plasma (226). Besides the incorporation of C and N, additional O was detected on the surface by X-ray photoelectron spectroscopy (XPS). In addition to the possibility of residual oxygen in the plasma reactor, two more explanations were proposed. First, the degradation of the superficial layer during plasma treatment may have led to volatized oxygen containing fragments, which recombined with the polymer surface. Second and more significantly, even after plasma polymerization, the surface of the film remained active so atmospheric oxygen and vaporized water were incorporated through radicalization. The high oxygen content could be reduced by pretreating the PHBV films with argon. The biocompatibility of PHBV was reported to be unchanged after the allylamine plasma polymerization. Nitschke *et al.* used ammonia for low-pressure plasma treatment of PHB (80). The nitrogen content of the surface increased nearly proportionally with increasing treatment time. The nitrogen was incorporated in the form of amide and amino groups. The contact angle decreased with increasing treatment. The hydrophilicity remained stable over the aging period of 6 weeks in the presence of air. The value of amino groups was demonstrated by covalently linking 4-trifluoromethyl benzaldehyde (TFBA) to the PHB films. Even though this reaction was designed to create a marker for the detection of amino groups by XPS, it showed that ammonia plasma treatment led to true functionalization of the surface of PHB. Amino groups can easily be used to bind biomacromolecules like peptides. Keen *et al.* introduced nitrogen functionalities to PHBV surfaces by ammonia plasma treatment and ethylenediamine aminolysis (ED) (227). In addition to amide and amino groups, evidence for imide groups was found in the XPS spectra of the treated samples. A treatment time of 5 or 10 s was sufficient to obtain optimal incorporation of desirable amino groups. Longer treatments resulted in higher nitrogen content without further increasing the proportion of amino groups. The plasma power did not affect the functionalization yield within the range of 2.5 to 20 W. In contrast, plasma power correlated to the wettability of the treated samples. As opposed to Nitschke and colleagues (80), Keen *et al.* described the wettability to be reversible within a short time period when exposed to air (227). However, the hydrophilicity could be regained by soaking the treated PHBV films in water. It was proposed that only the outermost molecular layer contained hydrophilic groups, which were buried in the presence of air. It should be noted that ammonia plasmas are unstable, however, they can be stabilized by adding an inert gas like argon to the plasma environment (228). Ethylenediamine aminolysis was reported to be disadvantageous compared to ammonia plasma treatment. PHBV films were treated with ethylenediamine in basic aqueous solution (pH 10) under constant stirring for various times up to 2 hours. Prior to subsequent material analysis, all samples were washed and vacuum-dried. Compared to plasma-treated films, fewer amino groups were found on ED-treated films. Moreover, crystallinity of ED-treated films increased over the time period of 3 weeks and also suffered from degradation during aging.

Oxygen and nitrogen plasma treatment were applied to PHBV films (229). The characterization of the surface properties was performed by water contact angle measurements and X-ray photoelectron spectroscopy. The wettability of the PHBV surface was improved as the water contact angle was reduced after plasma treatment. C-O and C-C bonds were found to be broken on the film surfaces. However, oxygen treatment led to the formation of carboxyl groups while exposure to nitrogen plasma resulted in nitrogen-enriched surfaces in the form of C-N, C=N, and amide bonds. The modified PHBV films were tested for adhesion of dog bone marrow stromal cells. In both cases, cell attachment was improved compared to cell adhesion on pure PHBV. There was no obvious difference in cell growth between nitrogen- and oxygen-treated PHAs. Therefore, both methods were suggested for the use of PHBV in tissue engineering (229).

6.3.2. Photografting

Grondahl *et al.* functionalized the surfaces of solvent-casted and melt-processed PHBV films by gamma-ray-induced grafting of acrylic acid (230). The biopolymers were soaked in methanol containing different concentrations of acrylic acid and then irradiated under nitrogen with doses ranging from 2 to 9 kGy. The presence of carboxylic groups on the surfaces, resulting from the successful photografting of acrylic acid, was demonstrated by binding pentafluorophenol to the reactive groups and by the uptake rate of toluidine blue, which functioned as a dyeing agent. While the radiation dose did not influence the functionalization yield of the melt-processed films, higher radiation doses favored the grafting reaction on solvent-casted PHBV surfaces. The density of the reactive functional groups could be controlled by the acrylic acid concentration in the UV radiation solution. The usefulness of polymer surfaces bearing carboxylic groups was demonstrated by immobilizing glycosamine on the PHBV polymers. Ke *et al.* photografted acrylamide onto PHBV using benzophenone as photoinitiator (231). Acetone was employed as a solvent for the 24 h photoinitiating step before the dried PHBV films were soaked in an aqueous acrylamide solution for the radiation treatment. Residual

acrylamide, polyacrylamide (PAM), and benzophenone were washed by Soxhlet extraction in acetone and rinsed in water. It was observed that both acrylamide concentration and irradiation time controlled grafting percentage (GP) and grafting efficiency (GE). The surface morphology changed notably as it became rougher with increasing GP. The increased number of acrylamide molecules and PAM did not only influence surface morphology. Attenuated total reflectance (ATR) and dispersive X-ray spectrometer (EDX) analysis revealed that radical PAM chains penetrated the polymers and even occurred on the other side of the PHBV films, forming a physical semi-interpenetrating network. Sheep bone marrow stromal cell studies showed that photografted PHBV films exhibited improved cell adhesion and cell compatibility. In a subsequent study, these grafted films were investigated for their wettability and crystallization behaviors (232). The water contact angle of PAM-grafted PHBV decreased to a minimum of 60 degrees from an initial 90 degrees. There was a direct correlation between GP and wettability. The crystallinity of the photografted PHBV films decreased with increasing GP, which promoted the diffusion of water into the polymeric structure.

Rasal *et al.* used a two-step photografting approach to tag the surface of PHBHx and PLA films with hydrophilic groups (233). In the first step, the polymeric films were dip-coated in a 5% (w/w) benzophenone solution in ethanol and irradiated with UV light after of ethanol had been removed by evaporation. After 5 min of radiation time, the treated films were sonicated in ethanol to remove residual benzophenone. During the second step, the benzophenone-grafted films were exposed to several hours of UV radiation in the presence of acrylamide and acrylic acid dissolved at 10% (w/w) in either ethanol or water. The twofold photografted polymers were again freed from residual non-grafted molecules by sonication. The contact angles were strongly reduced upon the introduction of acrylamide to the surface. PHBHx films exhibited a contact angle of 23 degrees after photopolymerization in ethanol and 28 degrees after the water-supported treatment. The contact angles on PLA films were decreased to 12 degrees and 17 degrees, respectively. While the choice of the photografted group affected the surface properties, the solvent employed for the second step impacted strongly on the bulk properties. When ethanol was used as a solvent for the second photografting step, acrylamide penetration into the bulk was observed. The surface-treated PHA and PLA films showed a significant loss in toughness and a gain in storage modulus, resulting in stiffer materials. This trend was not as severe when water was used as a solvent for carrying out the photopolymerization of acrylamide and acrylic acid on the surface of PLA and PHBHx.

6.3.3. Surface polarization for improved cell adhesion

The grafting of acrylamide onto PHBV films was induced using benzoyl peroxide (Bz_2O_2) (234). The reaction was carried out in water at 70 degrees Celsius under a nitrogen atmosphere. After precipitating the grafted polymer into methanol, it was washed in boiling water to remove any residuals. The graft percentage was significantly increased with increasing initiator and acrylamide concentrations. As a result, the polymeric films exhibited more polyacrylamide chains on the surface and became thicker. It was observed that radical graft polymerization did not only occur on the surface of the PHBV films. The swelling behavior of treated films in different solvent mixtures indicated that the polymers underwent a volume transition. Tesema *et al.* treated PHBV films with ozone to generate polar functional groups on the surface (235). The polymers were purged with ozone for 1 to 3 hours and the resulting peroxide groups were detected spectrophotometrically by the iodide method. The activated PHBV films were subsequently grafted with methacrylic acid (MAA) to obtain carboxylic groups that were used to chemically immobilize type I collagen on the film surfaces. Physically immobilized collagen was removed by sonication in deionized water. Peroxide formation during the ozone treatment was controlled by the treatment time and the concentration of ozone with which the films were purged. As expected, an increasing weight percentage of MAA monomers resulted in a higher carboxylic group concentration on the surface. Each of the modification steps carried out resulted in a decrease of the contact angle (from 59 to 33 degrees). In contrast, mean roughness peaked after ozonolysis before MAA grafting and collagen binding smoothed the surfaces. Untreated PHBV films, on which collagen had been physically immobilized, showed very similar values for contact angle and mean roughness. UMR-106 and MC3T3-E1 cell adhesion and proliferation tests revealed that collagen chemically immobilized PHBV films were the better material for supporting bone cell growth compared to collagen physically immobilized and untreated PHBV films.

Yang *et al.* (236) and Zhao *et al.* (237) exposed films made of PHB, PHBHx, PHBHx/PHB blends, and PLA to NaOH and lipase treatment. In both cases, hydrolysis of the ester bonds was expected to lead to free hydroxy groups. The attachment of mouse fibroblasts L929 was tested *in vitro* to check for changes caused by the surface treatment. Biocompatibility was improved in every case, however the positive effect decreased with increasing PHBHx content within the blend. The authors held the lower degree of crystallization responsible for a weaker lipase attack on the ester bonds. FTIR and scanning electron microscopy (SEM) analysis showed that lipase treatment also led to a change in surface morphology. According to Yang *et al.*, a reduced pore size promoted the adhesion of mouse fibroblasts (236). The effect of NaOH treatment was weaker compared to the one elicited by lipases (236, 237). Similar results were achieved by Zhao and colleagues (110). In a subsequent investigation, the biocompatibility of PHB and PHBHx lipase-treated scaffolds were compared to ones coated by hydrophilic hyaluronic acid (HA) (238). The assumption that a scaffold with the highest hydrophilic surface would lead to the best adhesion of mouse fibroblasts L929 was not confirmed. HA coating resulted in reduced cell growth. The authors therefore proposed that an optimal matrix surface would exhibit a combination of hydrophilic and hydrophobic area, instead of being completely hydrophilic. Moreover, this combination might vary from cell type to cell type. Surface hydrolysis was applied to PHBHx films to introduce

desirable functionalities to the surface (239). PHBHx films were soaked in 1M NaOH for 30, 60, or 90 min and extensively washed with deionized water. This surface etching led to the incorporation of hydroxyl and carboxyl groups. As a result, surface energy, and consequently wettability, increased. Moreover, the surface became rougher and, therefore, larger in size. The change in surface morphology of the PHBHx films resulted in an improved cell response owing to the introduced polarity and roughness. The adsorption of fibronectin was reported to be much higher than on unmodified films, while the attachment and proliferation of MC3T3-E1 cells was also significantly greater. A comparative study was performed, in which PHB and PHB4B were treated with NaOH solution as well as with NH_3 and H_20 plasma to improve the surface hydrophilicity of the polymers (240). The decrease in water contact angles was very similar on all treated PHA samples. Ammonia plasma treatment and ester hydrolysis due to NaOH resulted in a decrease of approximately 10 degrees. When water was used as plasma, the contact angle was reduced from 80.9 to 60.3 degrees (PHB) and from 79.3 to 62.5 degrees (PHB4B), respectively. Electrokinetic measurements confirmed the presence of carboxylic groups on the surfaces of NaOH and H_2O plasma-treated PHA films. In contrast, the isoelectric point was increased after the treatment with ammonia plasma, indicating the presence of alkalic amine functions. Fibronectin heteroexchange and cell adhesion were enhanced compared to untreated films and correlated with the physicochemical characteristics of the modified films.

Carboxyl ion implantation led to wettability improvement of PHB and PHBHx by causing surface destruction and formation of an amorphous carbon phase (241). Chen *et al.* implanted C^+ ions into PHB, PHBV, and PHBHx films (242). Biocompatibility was evaluated by seeding mouse fibroblasts 3T6 onto the implanted films. Cell attachment was improved after creating a newly formed hydrogenated carboneous layer on the surface. A high-energy ion-implantation machine was used to introduce hydroxyl ions to the surface of PHB films (243). Surface wettability was only slightly improved, however the bioactivity of treated PHB films was reported to be enhanced. Another way of introducing hydrophilicity to PHBV was achieved by fabricating PHBV/wollastonite composite scaffolds (244, 245). The evaluation of their bioactivity in simulated body fluid (SBF) led to the discovery that, after 14 days, the blended biomaterial had promoted the formation of a hydroxyapatite on the scaffold surface. Hydrophilicity increased with increasing wollastonite portion. Moreover, the calcium inosilicate mineral could neutralize acidic by-products that were generated during the soaking process in SBF by releasing Ca^{++} and SI^{++} ions and forming basic hydrates. This composite material could therefore be useful for bone tissue regeneration.

6.4. Other modifications

Inorganic-organic hybrid polymers were produced by covalently attaching polyhedral oligomeric silsesquioxanes (POSS) to PHOUs via a free radical addition reaction to the vinyl groups of the polymeric side chains (246). POSS chemicals were reported to result in property improvements when combined with polymeric materials, such as temperature and oxidation resistances (247) in addition to surface hardening and reduced flammability (248). The covalent linking was carried out by mixing mercaptopropyl-isobutyl-POSS (POSS-SH) and 2,2'-azoisobutyronitrile with PHOU (unsaturation degrees from 11.5 to 97%) in toluene under argon. The transformation of the double bonds to form PHOU-POSS derivatives resulted in conversion yields between 67.9 and 91.2%. All POSS-functionalized PHAs exhibited more heat stability as T_m and T_g were increased. The undesirable stickiness observed was successfully eliminated. The PHOUs underwent a change in crystallization behavior, resulting in a non-crystalline matrix for crystalline POSS.

Polyhydroxyalkanoates are of significant interest for biomedical applications due to their biodegradability and biocompatibility properties (9, 10, 38, 249, 250). Even though they may not be able to fulfill a desired task directly, they may contribute to do so as part of a composite material as indicated above. Further blending, grafting and copolymerization techniques as well as potential applications of resulting composite materials have been reviewed elsewhere (4, 10, 250-253).

7. CONCLUSIONS

Since the rediscovery of polyhydroxyalkanoates during the 1960's, the interest of researchers has increased to activate these bacterial storage compounds for human needs. For a long time, PHAs were believed to be inert and to exhibit poor thermo-mechanical properties, limiting their application spectrum. This changed drastically with the identification of bacteria, which are able to produce PHAs comprising medium-chain-length monomeric units (longer than C5). The possibility of tailoring their thermo-mechanical properties opened a new sub-area of research. Consequently, the potential to tailor the chemical structures by adjusting the feeding strategies created opportunities for biosynthetically introducing functional groups to PHAs. By the mid 1990's, the use of bacteriologically produced polyesters had changed from bioplastics with a limited application potential towards highly modifiable biomaterials with an increasingly diverse range of applications. The term functionalized polyhydroxyalkanoates was created.

Polyhydroxyalkanoates are currently strong candidates to play an important role in the development of second and third generation biomaterials, particularly in the field of tissue engineering. The natural production and degradation cycle of PHAs highlight their adaptability to living tissue. A number of modification techniques have been successfully applied to improve their thermo-mechanical and surface properties. Even though a large variety of functionalized PHAs have been biologically produced so far, many researchers agree that the production of polyhydroxyalkanoates bearing double bonds followed by chemical and/or physical modification is the more promising route to enhance the application possibilities of PHAs. Nevertheless, biopolyester activation within

microbial cells offers a significant advantage over other biomaterials.

The fact that PHAs can be used to produce materials for fine applications, which cannot be derived from petroleum, justifies their high production costs. In addition, due to the cost of fossil fuels and the increasing awareness of the consumer towards the environment, it is anticipated that the commercialization of polyhydroxyalkanoates as commodity plastics will continue to accelerate.

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Footnotes: [1] PHXY (m/n) = m mol% of X and n mol% of Y, [2] It is assumed that PHO in references (75, 99) and PHA in references (98, 101, 102) were PHOHx. The presence of 3HO without 3HHx is very unlikely.

Abbreviations: 3HB: 3-hydroxybutyrate; 3HHp(=): 3-hydroxyhept-6-enoate; 3HHx: 3-hydroxyhexanoate; 3HN(=): 3-hydroxynon-8-enonate; 3HO: 3-hydroxyoctanoate; 3HU(=): 3-hydroxyundec-10-enoate; 3HV: 3-hydroxyvalerate; AFM: atomic force microscopy; ATR: attenuated total reflectance analysis; CT: computed tomography; DBPH: 2,5-dimethyl-2,5-di(t-butyl peroxy) hexane; DMSO: dimethyl sulfoxide; DPTT: dipentamethylene thiuram tetra/hexa sulphide; DSC: differential scanning calorimetry; EB: electron beam; ECM: extracellular matrix; ED: ethylenediamine; EDX: X-ray spectrometer analysis; EGDM: ethylene glycol dimethacrylate; FTIR: Fourier transform infrared spectroscopy; GE: grafting efficiency; GGF: glial growth factors; GP: grafting percentage; HA: hyaluronic acid; HAP: hydroxyaptite; HMDA: hexamethylene diamine; Lcl: long-chain-length; MAA: methacrylic acid; Mcl: medium-chain-length; MCPBA: m-chloroperbenzoic acid; MRI: magnetic resonance imaging; NMR: nuclear magnetic resonance spectroscopy; PAM: polyacrylamide; PBS: phosphate buffer solution; PCL: polycaprolactone; PEO: poly(ethylene oxide); PFH: perfluorohexane; PGA: polyglycolic acid; PH4B: poly(4-hydroxybutyrate); PHA: polyhydroxyalkanaote; PHA-L: PHA made from linseed oil; PHA-tal: PHA made from tallow; PHB: poly(3-hydroxybutyrate); PHB4B: poly(3-hydroxbutyrate-co-4-hydroxybutyrate); PHBO: poly(3-hydroxbutyrate-co-3-hydroxyoctanoate); PHBV: poly(3-hydroxbutyrate-co-3-hydroxyvalerate); PHO: poly(3-hydroxyoctanoate); PHOHx: poly(3-hydroxyoctanoate-co-3-hydroxyhexanoate); PHOHxD poly(3-hydroxyoctanoate-co-3-hydroxyhexanoate-co-decanoate); PHOU: poly(3-hydroxyoctanoate-*co*-3-hydroxyundec-10-enoate); PHU: poly(3-hydroxyundec-10-enoate); PLA: polylactic acid; POSS: polyhedral oligomeric silsesquioxanes; SBF: simulated body fluid; SC: Schwann cells; Scl: short-chain-length; SCPL: solvent casting and particle-leaching; SEM: scanning electron microscopy; TFBA: 4-trifluoromethyl benzaldehyde; Tg: glass transition temperature; Tm: melting temperature; TPO: diphenyl(2,4,6-trimethylbenzoylphosphane oxide); UV: ultraviolet; XPS: X-ray photoelectron spectroscopy

Key words: Functionalized polyhydroxyalkanoates, biopolyesters, tissue engineering materials, biomaterials, biocompatibility, biodegradability, thermo-mechanical properties, biosynthesis and production, chemical synthesis, chemical, physical and surface modifications, curing, plasma polymerization, photografting, review

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