CHAPTER 7

Modification of Polyhydroxyalkanoates (PHAs)

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7.1 Introduction

Polyhydroxyalkanoates (PHAs) are biodegradable and biocompatible polyesters with versatile structural compositions. Bacterial PHAs are produced using a combination of renewable feedstock and biological methods mostly *via* a fermentation process. Native and recombinant microorganisms have been generally used to produce different types of PHAs, such as homopolymers[1,2] and copolymers of diverse morphology.[3–5] Alternative production schemes of PHAs *in vitro* based on cell-free enzymatic catalysis are gaining momentum and may become the preferred route to some specialty products.[6,7]

In addition to their biodegradability, compatibility, and compostability, PHAs were reported to possess gas-barrier properties almost similar to those of polyvinyl chloride and polyethylene terephthalate.[8] These combinations of excellent physico–chemical properties coupled with the current concerns over environmental pollution and waste degradation drive their increasing commercial exploitation in different niche applications spanning from biomedical, packaging, automotive, infrastructure, aerospace to military applications.[7,9]

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Unfortunately, despite their high potential for commercial applications, most of the PHAs produced, especially those belonging to crystalline short chain length PHAs or medium chain length PHAs with higher monomeric compositions of 3-hydroxybutyric acid, were said to be highly hydrophobic, and exhibited brittleness, a low heat distortion temperature and poor gas-barrier properties. This resulted in slow degradability and resorbability within the extracellular matrixes as well as limited malleability and ductility during industrial processing.[10,11] As such, these kinds of neat biopolymers fail to meet the industrial demands especially in aggressive environments. Therefore, several approaches were devised to enhance the physico–chemical properties of the PHA in order to overcome these shortcomings by modifying the biopolymers through different processes.[9]

For example, metabolic engineering and culture condition manipulation were employed to produce modified PHAs with salient features. PHAs with methyl side-chains such as PH6N (poly(3-hydroxy-6-methyl-nonanoate)) was obtained from *Pseudomonas oleovorans* fed with methylated alkanoic acids or in a mixture with nonanoic acid as a carbon source.[12] PH6N crystallizes much faster than neat PHN (poly-3-hydroxynonanoate),[13] and the melting temperature was higher ($T_m = 65\ ^\circ C$) than that of PHN ($T_m = 58\ ^\circ C$).[14] Studies have shown that PHAs containing an epoxidized group were accumulated inside *P. oleovorans* when fed with octanoate and 10-undecenoic acid[15] and inside *P. cichorii* YN2 when fed with 1-heptene to 1-dodecene as a sole carbon source.[16] Alternatively, *P. oleovoran*s accumulated sulfanyl PHA upon feeding with *m*-(*n*-thienylsulfanyl) alkanoic acid.[17] Furthermore, the bacteria was found to accumulate brominated PHA when fed with a mixture of ω-bromoalkanoic acids and nonanoic or octanoic acid.[18] Changing the carbon source to octane and 1-chlorooctane or nonanoic acid and fluorinated acid co-substrates resulted in the accumulation of chlorinated or fluorinated PHA in *P. oleovorans,* respectively.[19,20] Feeding propylthiooctanoic acid (PTO) or propylthiohexanoic acid to metabolically engineered *Ralstonia eutropha* led to the production of thiol functionalized PHA with enhanced chemical properties.[21] On the other hand, addition of low molecular weight diols such as ethylene glycol to the fermentation medium resulted in the bacterium producing a bi-functional telechelic hydroxyl-terminated PHA.[22,23]

The presence of these types of functional groups in specialized PHAs allows for further functional group modification. However, such bio-synthetic approaches could only produce the specified polymer in minute quantities and most of the time, the whole-cell metabolic framework of the fermentation process itself limits the degree of freedom in designing the PHA with other functional groups of interest. As such, alternative routes for modification need to be applied. In order to extend their applications in aggressive environments where the neat polymers have failed, modification (functionalization) of the polymer *via* chemical,[24] physical[25] or enzymatic[26] processes have been employed. For example, sugars with pyranose structures, such as galactose and mannose, were reported to be ligands specific

to the over-expressed asialoglycoprotein receptor (ASGPR) in hepatocellular carcinoma.[27] Hence, modifying the PHA *via* attachment with these types of sugars could help to enhance the cancer drug targeting potential of the modified material.[28] Similarly, chemically modified PHA with folic acid could efficiently serve as a cancer drug carrier by targeting folate receptor (FR), a glycosylphosphatidylinositol anchored protein, which appears to be up-regulated in more than 90% of non-mucinous ovarian carcinomas.[24]

Modification of PHA pendant groups was reported to enhance the degradation rate of the polymer.[29] For example, converting the pendant groups to carboxylic acids or basic amine groups enhances PHA degradation by pH alteration. On the other hand, pendant groups converted to hydrophilic groups (2-hydroxy acids *e.g.* glycolic and lactic acid) increase the uptake of hydrolytic agents such as water or a physiological buffer. Moreover, the pendant groups may also be converted to groups that would increase the polymer's porosity using active compounds such as inorganic salts and sugars that are removed by leaching. In addition, modifying PHAs by direct fluorination was reported to cause a marked change in their thermo–chemical properties thereby extending their niche applications.[30] Other reactive groups, such as amines, alcohols, amino acids and amino alcohols, and multifunctional monomers such as triols and tetraols, were also used as polymer pendant modifiers that can subsequently participate in polymer intra- or inter-molecular modifications.[29] As well as expanding their applications, modifications to PHAs also increase the availability of different types of biologically produced polymers. This chapter discusses the different methods of PHA modification.

7.2 Chemical Modification of PHAs

The structure of a PHA can be altered chemically to produce a modified polymer with predictable variation in molecular weight and functionality. For example, the hydrolytic rate of PHA to give an activated macromer that can further accept a reactive functional group is said to depend on several factors such as the chemical nature or reactivity of the ester linkages between the monomers.[29] Thus, the hydrolytic rate can be modified to become relatively fast by incorporating more hydrolysis-prone chemical groups (*e.g.* hydroxy-acids such as 2-hydroxyethoxy acetic acid, amide, anhydride, carbonate, carbamate *etc*) into the polymeric backbone thereby modulating the reactivity of the ester linkage.[23]

Unlike PHA modification *via* manipulation of the fermentation process, chemical reactions allow for the bulk production of a uniform product and incorporation of diverse functional groups to produce useful tailor-made polymers with desirable properties for niche applications. PHA modification by chemical processes can be achieved by different methods such as carboxylation, hydroxylation, epoxidation, chlorination, grafting reaction *etc*. (Figure 7.1).[14,31]

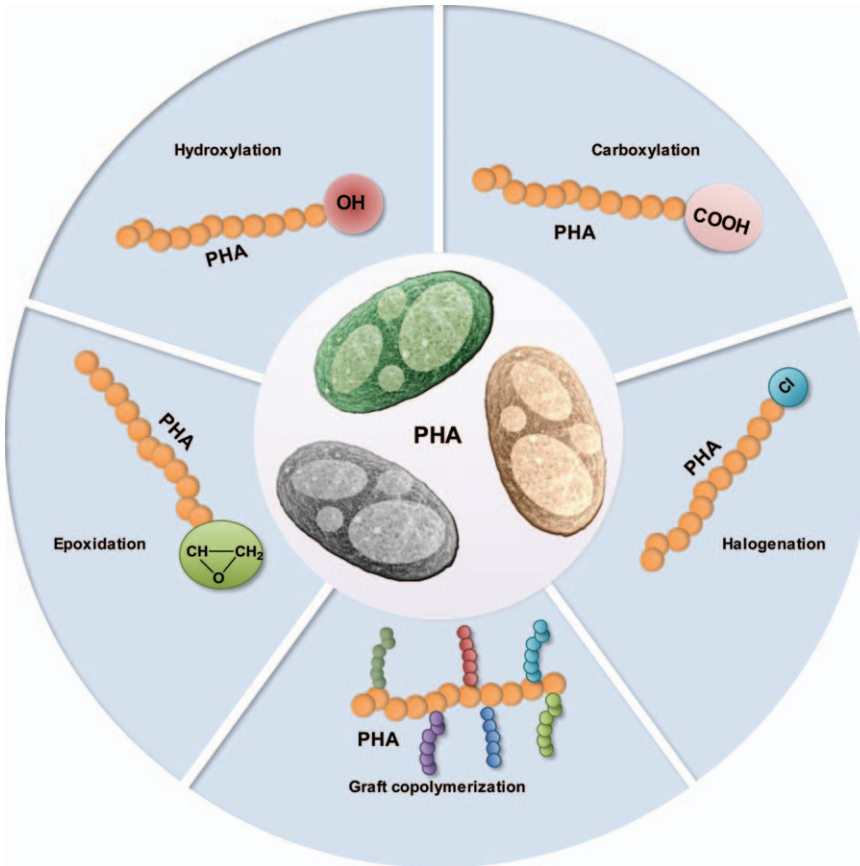

Figure 7.1 Typical methods for the chemical modification of PHAs to yield different types of functionalized polymers.

7.2.1 Carboxylation

PHA modification by carboxylation is the addition of carboxylic functional group to the polymeric macromer (Figure 7.2). Carboxylic groups incorporated into the polymer usually serve as functional binding sites for bioactive moieties such as probes for targeting proteins and hydrophilic components.[31] Studies have shown that the carboxylation of PHA enhances the hydrophilicity of the polymer through better water penetration, which is encouraged *via* ester group hydrolysis by water.[32–34]

A chemical oxidative process is normally employed to carboxylate PHA with an unsaturated group.[14,31,33] Previously, de Koning *et al.*[35] carboxylated the double bond of poly-3-hydroxyoctanoate-*co*-3-hydroxyundecenoate (PHOU) using $KMnO_4$ as an oxidation agent in the presence of $NaHCO_3$. This method was further modified by Kurth *et al.*[34] and Lee and Park[32] where crown ether was used as the phase transfer and dissociating agent for the

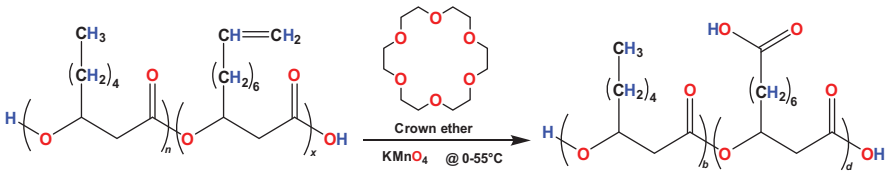

Figure 7.2 Oxidative carboxylation of unsaturated PHA using $KMnO_4$ as an oxidation agent.

$KMnO_4$ to carboxylate the unsaturated PHOU (Figure 7.2). The authors were able to quantitate the oxidation based on the disappearance of the ^{1}H NMR peak ($\delta = 4.9$ and 5.7 ppm) assigned to the double bond.[34] Although a complete oxidation of the double bonds was achieved, the researchers found that 25% carboxylation of PHOU was sufficient to enhance the hydrophilicity of the polymer.[25] Furthermore, they reported that the carboxylated polymer suffered a major loss of molecular weight compared to neat PHOU.[32,34] The decrease in the molecular weight was attributed either to macromolecular chain degradation during the process or as a result of differences in hydrodynamic radii between the carboxylated polymer and SEC polystyrene standards.[34] Lee and Park[32] reported a reaction time of 2 h as optimal to achieve maximum carboxylation of 50%, where a modified PHA with a degree of carboxylation between 40–50% was observed to be completely soluble in water/Na_2CO_3.[32]

In an attempt to address the issue of molecular weight degradation upon carboxylation, Stigers and Tew[36] developed a new synthetic process that uses osmium tetraoxide and oxone in dimethylformamide. The oxidation reaction took place at 60 °C for 8 h, and proceeded to completion with little polymer degradation.[36] The carboxylic group in the modified PHA proved advantageous in producing block and grafted copolymers. Condensation reactions between carboxylic acids and amine groups were exploited to graft modified PHA and linoleic acid onto chitosan.[37] Recently, Babinot *et al.*[38] used click ligation to esterify the pendant –COOH of carboxylated PHA with propargyl alcohol resulting in a clickable-alkyne group that was subsequently used to copolymerize poly(ethylene glycol) (PEG) macromer onto the modified PHA.

7.2.2 Hydroxylation

The properties of PHA and its copolymers have been reported to be modified by hydroxylation.[39–42] Normally, acid- or base-catalyzed reactions are used in the modification of PHA by hydroxylation in the presence of low molecular weight mono- or diol compounds (Figure 7.3). Hydroxy-terminated PHA is of importance in block copolymerization. Methanolysis of PHA resulted in PHA methyl esters bearing monohydroxy-terminated groups.

Timbart *et al.*[43] reported the production of monohydroxylated oligomers of poly-3-hydroxyoctanoate (PHO) and PHOU using both base- and

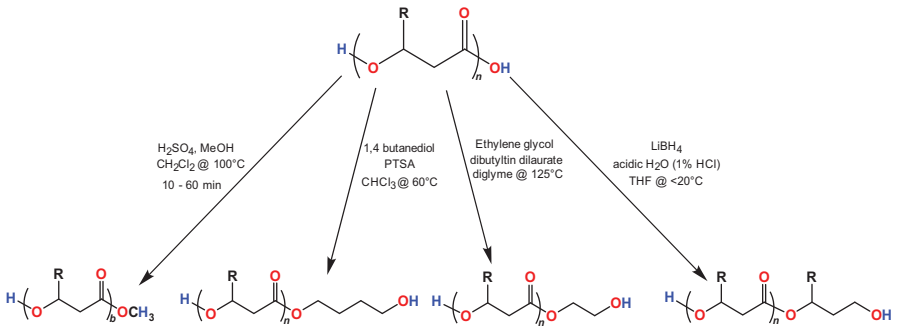

Figure 7.3 Different approach to PHA modification by hydroxylation; R = alkyl side chain.

acid-catalyzed hydrolyses. In basic hydrolysis, the researchers used alcoholic NaOH to catalyze the hydrolysis at pH 10–14 and the reaction was stopped upon addition of concentrated aqueous hydrochloric acid. On the other hand, the acid hydrolysis was performed using two different approaches *viz*. (i) a reaction catalyzed by *para*-toluenesulfonic acid monohydrate (PTSA) at 120 °C, which was stopped by cooling the mixture in an ice bath; (ii) monohydroxylation by acidic (sulfuric acid) methanolysis at 100 °C to yield the respective 3-hydroxymethyl esters bearing a methyl protected carboxylic acid group. It was also reported that the kinetics of PHO oligomer production depends on the reaction conditions. The ester bonds of PHO were shown to be stable within pH 10–12. Increasing the pH to 14 resulted in a higher hydrolysis rate and produced oligomers with unsaturated end groups due to a McLafferty rearrangement.[43] The researchers concluded that an acid-catalyzed reaction and methanolysis were more efficient than basic hydrolysis in producing PHO oligomers. They also showed that the observed decrease in polymer molecular weight was dependent upon the solvent type used. Polymeric chain cleavage was observed to occur more frequently in toluene than in dichloroethane, an observation that was attributed to the better solubility of the polymer in toluene. In another study, *p*-toluenesulfonic acid (PTSA) in the presence of methanol was used to catalyze the monohydroxylation of poly-3-hydroxybutyrate-*co*-4-hydroxybutyrate (P3HB4HB).[39] The modified PHA was acrylated and grafted onto poly(ethyleneimine) *via* Michael addition, which resulted in a material that was used to deliver siRNA (Figure 7.4).[39]

In the preparation of PHA tri-block copolymers, a bi-functional telechelic PHA as a macro-initiator is usually required. In this process, low molecular weight diols are used as the micro-initiator of the PHA-diol reaction. During the reaction, the hydroxyl groups of the diol were proposed to cleave the polymeric ester bonds in a random fashion, resulting in the dihydroxy terminated PHA.[31] Dibutyltin dilaurate was used as a chemical catalyst in the presence of ethylene glycol (micro-initiator) to produce an enantiomerically pure telechelic dihydroxy-terminated PHO and its

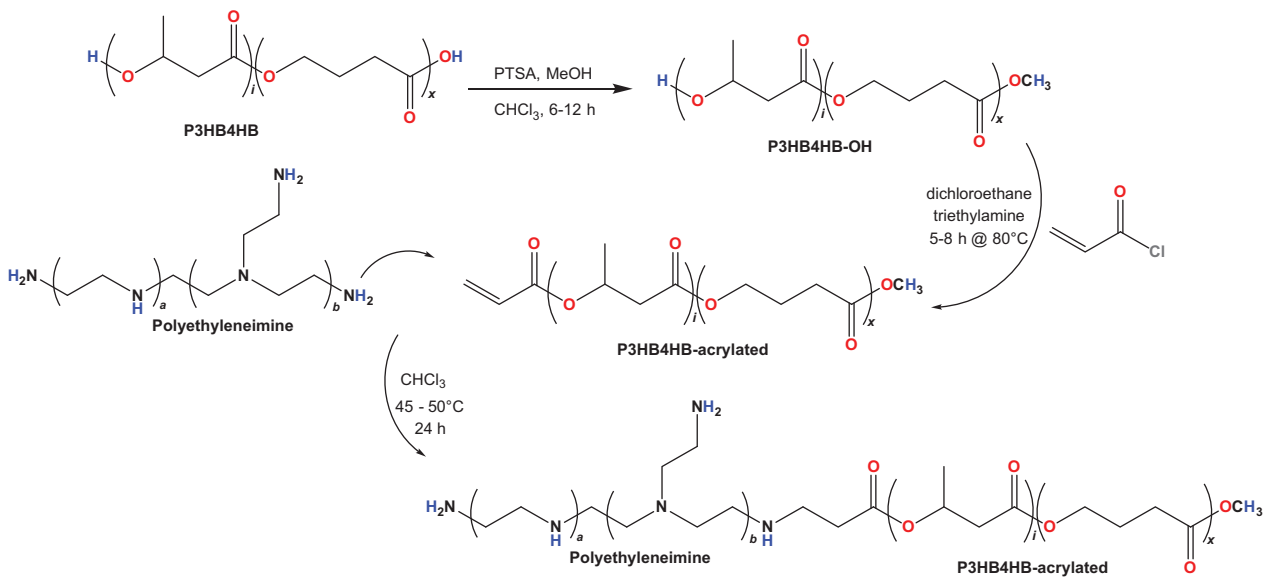

Figure 7.4 PHA modification by monohydroxylation and subsequent graft polymerization by Michael addition.

copolymers at 80–91% yield.[42] It was found that the modified telechelic PHO-diol showed lower glass transition (T_g) and melting (T_m) temperatures than neat PHO. However, this observation was found to contradict the reported increase in the thermal stability of di-hydroxy terminated P3HB synthesized by microbial fermentation.[23] Using 1,4-butanediol and PTSA, Chen *et al.*[44] produced modified P3HB4HB and poly-3-hydroxybutyrate-*co*-3-hydroxyhexanoate (PHBHHx) diols that were grafted with poly(ester-urethanes) *via* Michael addition. Recently, Kwiecień *et al.*[40] described a new method for P3HB4HB hydroxylation by selective partial degradation of the polymer ester bonds using lithium borohydride. The reaction was carried out by drop-wise addition of 2 M $LiBH_4$ at a temperature <20 °C after the polymer was completely solubilized in tetrahydrofuran (THF). After a series of purification steps of chloroform and water washing followed by evaporation of the organic phase, about 97% of purified modified PHA oligodiols were obtained. As revealed by NMR and electrospray ionization-mass spectrometry (ESI-MS) analyses, the applied reduction process of the PHA oligodiols was highly selective.[40] The researchers claimed the method to be versatile and applicable to the production of any PHA oligodiol. Block copolymers of PHO and poly(ester-urethanes) were synthesized by a reaction between the polymer hydroxyl group and the –NCO group of aliphatic *L*-lysine methyl ester diisocyanate (LDI) that was employed as a junction unit.[45] The aliphatic diisocyanate (LDI) was chosen over the aromatic diisocyanate due to the absence of the toxic, mutagenic, and carcinogenic aromatic amine degradation byproducts of the aromatic diisocyanate derived polyurethane. Similarly, Chen *et al.*[46] used facile melt polymerization to synthesize a block poly(ester-urethane) based on diols of P3HB4HB and poly-3-hydroxyhexanoate-*co*-3-hydroxyoctanoate (P3HHx3HO) using 1,6-hexamethylene diisocyanate (HDI) as the coupling agent. Lactate dehydrogenase (LDH) assay and platelet adhesion determination revealed that the synthesized material showed much higher platelet adhesion than the neat polymers, and even higher than that of polylactic acid (PLA) and poly(3-hydroxybutyrate) (PHB).[46] In fact, this type of poly(ester-urethane) was reported to possess the properties required for use in medical applications including enhanced wound healing activity.[46] A large group of PHA-based materials were produced from hydroxylated PHA, and have been reviewed extensively.[31]

7.2.3 Epoxidation

The high reactivity of epoxide groups under mild conditions was described as an important factor in medium-chain-length (mcl) PHA modification (Figure 7.5(a)). The epoxide group can participate in diverse reactions such as cross-linking (Figure 7.5(b)) to attach copolymers, bioactive moieties, or an ionizable group without unwanted polymer degradation.[31,47] Several studies have reported successful chemical modification of PHA by epoxidation.[48,49]

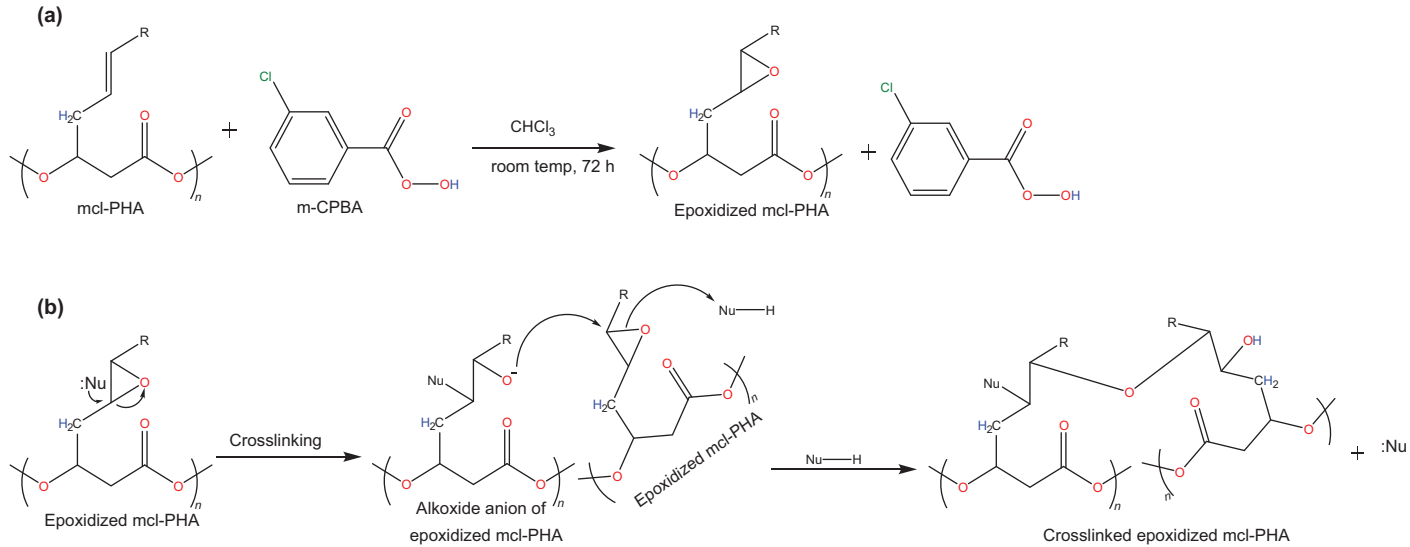

Figure 7.5 (a) Epoxidation of mcl-PHA using *m*-CPBA; (b) ether cross-linking of the epoxidized mcl-PHA.

A study by Bear *et al.*[15] reported the chemical epoxidation of mcl-PHA obtained from *P. oleovorans* and *Rhodospirillum rubrum* using *m*-chloroperoxybenzoic acid (*m*-CPBA). Proton NMR analysis revealed about 36.7% epoxidation that was calculated upon comparing the α,β-oxirane proton signals (2.75 and 2.9 ppm) with the signals of methylene protons (2.6–2.5 ppm) in the PHA backbone. Since Bear *et al.*'s report on the chemical epoxidation of PHA, there have been several reports on a similar epoxidation process. Park *et al.*[48] epoxidized PHOU with a controlled amount of olefinic bonds using *m*-CPBA.[48] Regardless of the number of polymeric olefinic groups, the researchers observed the process to follow second-order reaction kinetics with an observed initial reaction rate (v) of 1.1×10^{-3} L mol^{-1} s^{-1} at 20 °C. However, both the melting temperature (T_m) and melting enthalpy were observed to decrease with increasing conversion of the olefinic bonds to epoxy groups. Interestingly, the authors reported an increase in glass transition temperature (T_g) by about 0.25 °C for each 1 mol% of epoxide group, irrespective of the PHOU composition used. In similar studies, the researchers cross-linked the epoxidized PHOU with succinic anhydride in a reaction initiated by 2-ethyl-4-methylimidazole and carried out at 90 °C for a period of 0.5 to 4 h, which resulted in a highly elastic cross-linked PHA.[49] In their study, they found that by carrying out the reaction in mild acidic conditions, the reported polymer degradation was inhibited. The cross-linking kinetic parameters were evaluated using Kissinger[50] and Ozawa[51] models, and they calculated a cross-linking activation energy of 15.6–16.0 kcal mol^{-1} in all the reactions.[49] When evaluating the thermal stability of the epoxidized PHA, Park *et al.*[52] observed that the polymer thermal stability increases with increasing epoxy group. The observed increase in the thermal stability was attributed to intermolecular thermal cross-linking reactions between the pendant epoxy groups and the carboxylic acid groups generated from the polymer random chain scission by β-elimination (Figure 7.6). This interpretation was derived from the appearance of thermal exothermic peak ''b'' (375 °C) normally associated with a cross-linked reaction, followed by the endothermic melting temperature peak ''a'' (299 °C) in the differential scanning calorimetric (DSC) thermogram of the epoxidized polymer (Figure 7.7).

Mcl-PHA obtained from linseed oil was reported to possess a high number of olefinic side-chains, making the polymer consistently viscous and sticky at room temperature.[53] The polymer has limited potential applications, except as a bio-adhesive, but the range of applications can be expanded by improving the rigidity and stiffness of the polymer. Ashby *et al.*[53] used *m*-CPBA (as illustrated in Figure 7.5(a)) to convert about 37% of olefinic bonds in linseed oil derived mcl-PHA side-chains to epoxy groups in order to enhance the mcl-PHA cross-linking ability.

Comparing the ^{13}C NMR spectra of both the neat mcl-PHA (Figure 7.8(a)) and the epoxidized mcl-PHA (Figure 7.8b), the researchers confirmed the polymer epoxidation by the appearance of an epoxide chemical shift at 58 ppm as shown in Figure 7.8(b). They explained that steric hindrance

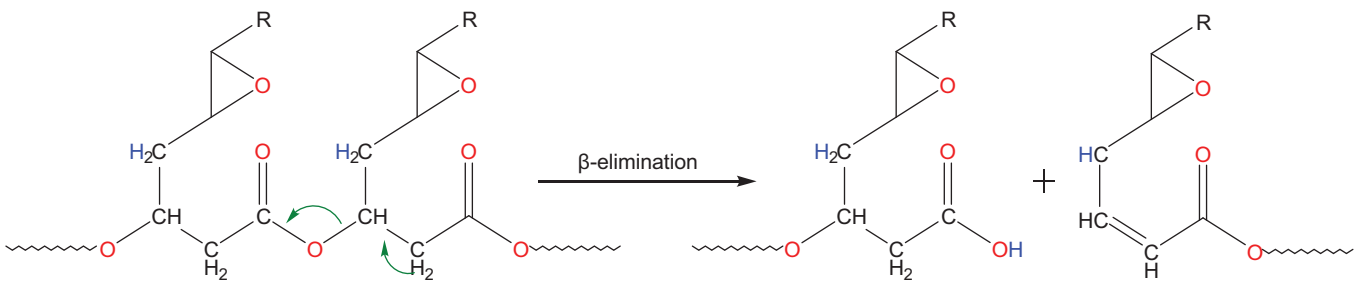

Figure 7.6 Generation of carboxylic acid from polymer random chain scission by β-elimination.

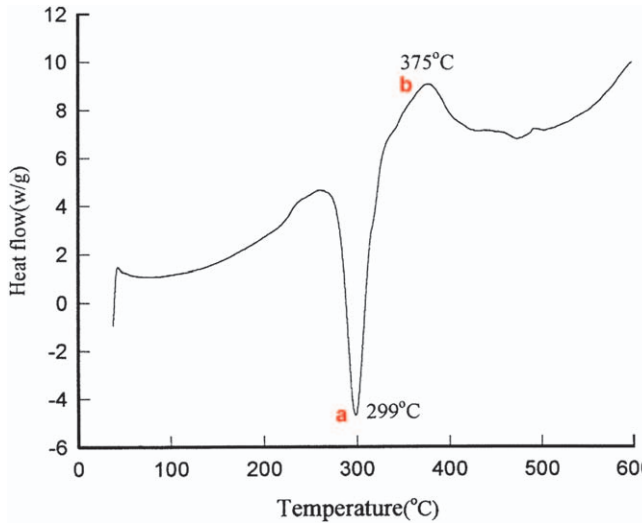

Figure 7.7 DSC thermogram for epoxidized PHO showing an endothermic melting peak (a) and exothermic cross-linking peak (b) reproduced from Park *et al.*[52] with permission from Elsevier.

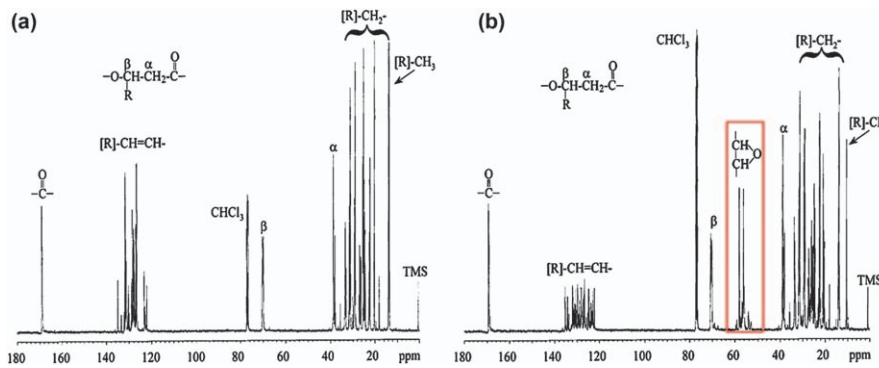

Figure 7.8 ^{13}C NMR spectra of linseed derived mcl-PHA (a) neat polymer (b) epoxidized polymer, reproduced from Ashby *et al.*[53] with permission from Elsevier.

caused by the proximity of the internal olefins to the side-chains to be the reason behind the low yield of olefinic conversion. The intermolecular cross-linking was suggested to have occurred *via* ether cross-links as depicted in Figure 7.5(b). In this type of cross-linking, it was proposed that a nucleophile (Nu) or radical species first opened the carboxy-oxirane ring leading to re-arrangement of electrons and the formation of an alkoxide anion, which in turn initiated a nucleophilic attack on another epoxidized PHA oxirane carbon (Figure 7.5(b)).[53]

The study showed that the epoxidation enhanced the cross-linking of the mcl-PHA when exposed to air, resulting in an increased tensile strength and Young's modulus from 4.8 to 20.7 and 12.9 to 510.6 MPa, respectively. Consequently, this conferred short-term (25 days) stiffness to the modified PHA compared to a long period (50–75 days) for the corresponding neat PHA.[53]

In another study, Lee *et al.*[54] reported a successful cross-linking of epoxidized PHO using hexamethylene diamine (HMDA) as a cross-linker in a reaction carried out at 90 °C for 0.5 to 24 h (Figure 7.9). The degree of cross-linking was found to significantly influence the T_g and relative storage modulus of the modified copolymer. In addition, the effect of cross-linking on the thermal stability of the epoxidized PHA was studied by cross-linking the PHA with either succinic acid (in the presence of a catalyst) or HMDA (in the absence of a catalyst).[55] The study showed that the increment in the amount of basic catalyst or diamine cross-linker caused a decline in the thermal stability of cross-linked PHA.[55] The observation was attributed to ester cleavage of the polymer backbone catalyzed by the basic catalyst or cross-linker amine groups.

7.2.4 Halogenation

Halogenation of PHA is considered an excellent method in diversifying the polymer functions and applications. Halogen atoms such as chlorine, bromine and fluorine were added to the olefinic bonds of unsaturated PHA through an addition reaction,[56] and to the saturated PHA *via* substitution reactions.[57] Excess HCl was added to $KMnO_4$ in a drop wise fashion to generate chlorine gas (Figure 7.10). The gas was subsequently passed into a solution of sticky unsaturated PHA obtained from *P. oleovorans.* This resulted in a stiff and crystalline polymer at approximately 54 wt% chlorination.[56] Depending on the chlorine content, the chlorinated PHA (PHA-Cl) exhibited higher melting and glass transition temperatures ($T_m = 125$ °C, $T_g = 58$ °C) compared to neat PHA ($T_m = 55$ °C, $T_g = -50$ °C).[56] However, an observed hydrolysis of the polymer backbone was also reported due to the reduced molecular weight of the PHA-Cl with increasing chlorine content.[56]

In another study, Arkin and Hazer[57] modified the PHA-Cl into quaternary ammonium salts, thiosulfate moieties and phenyl derivatives. In addition, they cross-linked the modified PHA-Cl with benzene by electrophilic aromatic substitution using a Friedel–Crafts reaction. The random composition of PHA-Cl was calculated from its ^{1}H NMR spectrum by comparing the relative peak areas of the methine protons on the polymer backbone. Hence, increased chlorination of the methyl protons caused the peak of methine protons to be moved further downfield. In addition, the PHA-Cl mole fractions were calculated by comparing the peak areas of protons on chlorinated α-carbons and protons on β-carbons.[57] Samsuddin *et al.*[30] described a process for the direct fluorination of PHBHHx at elevated pressure in the

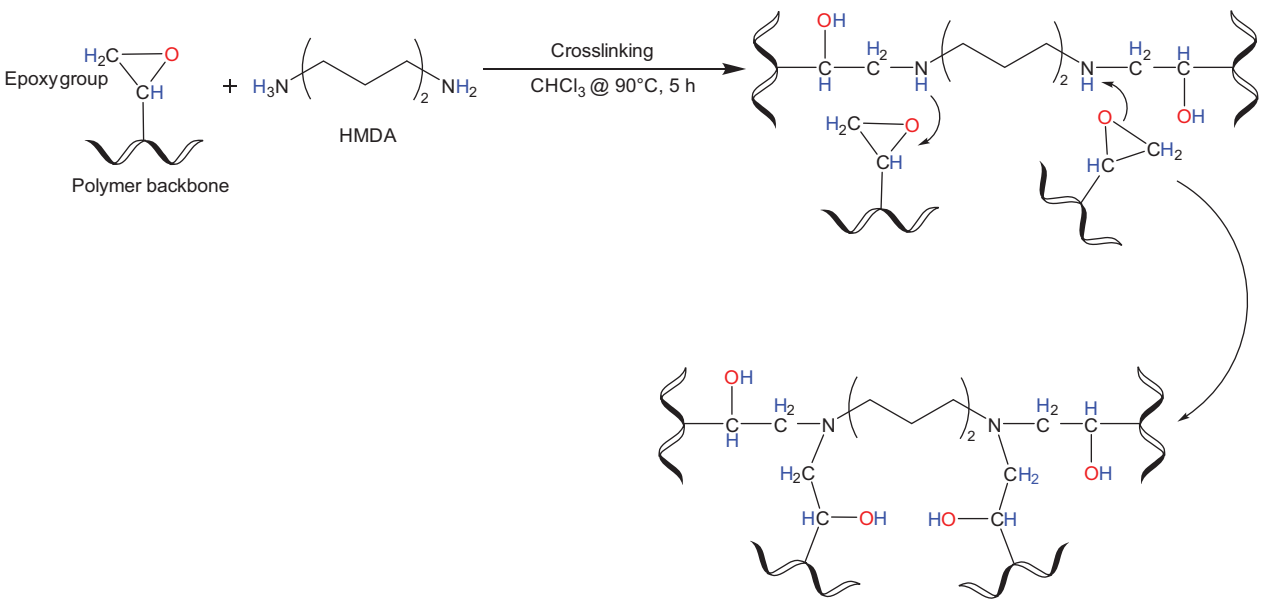

Figure 7.9 Cross-linking of epoxidized mcl-PHA using hexamethylene diamine (HMDA) as a cross-linker.

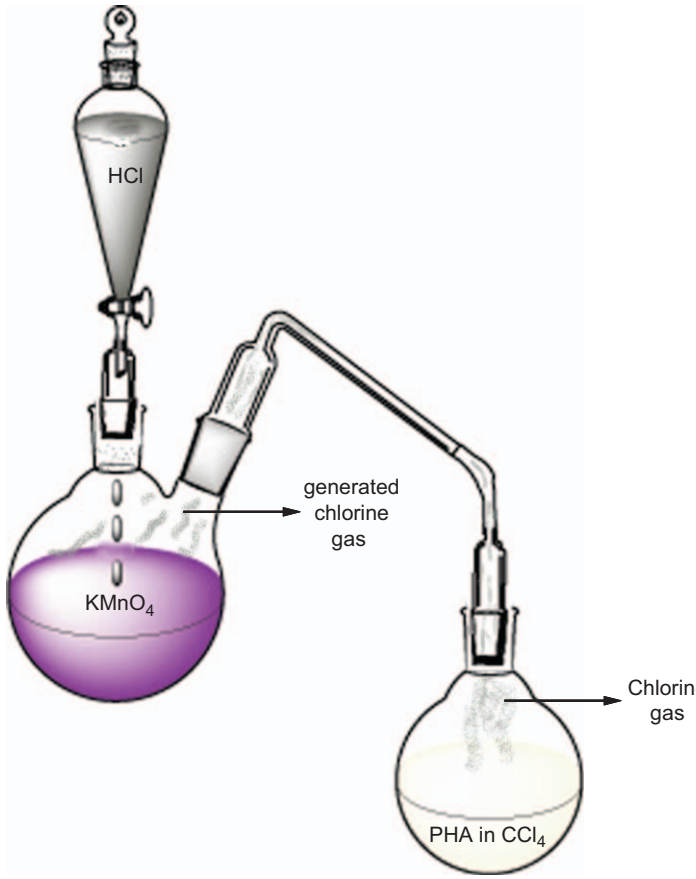

Figure 7.10 Reaction set-up for the chemical modification of PHA *via* chlorination.

presence of an elemental F_2/N_2 gas mixture. The fluorinated polymer was reported to show marked changes in its thermo–chemical characteristics.[30]

Mihara *et al.*[58] and Imamura *et al.*[59] filed an embodiment that detailed the procedures for PHA chemical modification by sulfanyl halogenation and the potential application of the modified PHA as a toner electrostatic charge controller in electrophotographic imaging. The inventors disclosed that the modified PHA possesses excellent charging stability, high charging ability, and enhanced dispersibility, when used as a toner charge control agent in an electrophotographic process.[58,59]

7.2.5 Graft and Cross-linking Polymerization

Another method to modify PHA is by graft copolymerization, which results in the formation of a modified segmented copolymer with improved properties such as increased wettability and thermo–mechanical strengths.

Grafting reactions can be induced by either chemical, radiation or plasma discharge methods.[60,61] Among the different approaches used in chemical graft copolymerization, the ''grafting through'' approach is by far the simplest process. The approach involves copolymerizing the PHA macromer with low molecular weight monomer by atom transfer radical polymerization to obtain a well defined copolymer with improved physico–chemical properties. Lao *et al.*[62] grafted 2-hydroxyethylmethacrylate (HEMA) onto poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBHV) using benzoyl peroxide (BPO) as a micro-initiator. The study revealed that grafting HEMA onto the PHA helped to improve the crystallinity and wettability of the polymer. Similarly, methyl methacrylate (MMA) was thermally grafted onto olefinic PHA derived from soybean oil using BPO as a chemical initiator.[63] A fractional precipitation method was used to isolate the graft copolymer from the reaction mixture of related homopolymers. The material was stored in hydroquinone to prevent intermolecular post polymerization by cross-linking. The modified polymer was shown to exhibit improved thermo–mechanical properties.[63] Higher antimicrobial activity and biocompatibility were imparted into PHO by grafting with vinylimidazole using the same thermal grafting approach in the presence of BPO initiator, resulting in a modified polymer (VI-*g*-PHO). The grafted polymer, when tested at 2% (w/v) suspension, showed a >90% reduction in viable cell counts against *Escherichia coli, Staphylococcus aureus* and *Candida albicans*.[64] Similarly, an ozone-treated PHBV membrane was grafted with acrylic acid that was subsequently esterified with chitosan. A collagen immobilized membrane was shown to improve L929 murine fibroblast cell proliferation. It also exhibited enhanced antimicrobial activity against *Staphylococus aureus, Escherichia coli* and *Pseudomonas aeruginosa*.[65] Application of PHA in neoglycoconjugate reactions helps to improve its hydrophilicity, degradability and bioresorbability. The conjugated PHAs were reported to be highly important in biological studies such as tissues and stem cell research.[66] Anti-Markovnikov addition to the double bond of unsaturated PHA was used to graft maltosyl units resulting in a hydrophilic grafted copolymer that is readily soluble in dimethylformamide and dimethyl sulfoxide but insoluble in dichloromethane and chloroform.[67] When acrylic acids were grafted onto ozone modified PHB and PHBV, followed by grafting of chitosan or chitooligosaccharide *via* esterification,[68] the modified PHA showed a strong antibiotic activity against *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA). In addition, acrylic acid grafting alone increases the biodegradability, whereas grafting with chitosan or chitooligosaccharide reduces the biodegradability. In contrast to the chitosan-grafted-PHBV membrane, the chitoooligosaccharide-grafted-PHBV membrane showed lower antibacterial activity but higher biodegradability.[68]

Diazo linkage derived from diazo polyesters of ethylene glycol and ethylene glycol methacrylate were used as macro initiators in the process of forming different graft copolymers that were used in smart hydrogels for

biomedical applications.[69–72] Previously, the elastic response of thermoplastic polyhydroxyalkanoates (PHOU) was improved upon cross-linking the side-chain olefinic bonds using peroxide as a cross-linker.[73,74] The study showed that the degree of cross-linking is a function of the type of peroxide and its concentration, and the cross-linking improved the elastic response by reducing the material crystallinity and tensile strength.[73,74]

7.3 Physical Modification of PHAs

The diverse potential applications of PHA in a number of fields demanded the production of smart polymers with minimal toxic impurities. Chemical modification methods are sometimes aggressive, and lead to reduced polymer molecular weight, unwanted side reaction(s) and toxic impurities. In some instances, a mild surface modification process is required without which the polymer may fail in its intended application(s). For example, neat polymer without the proper modification may cause delamination of adhesive bonds, poor cellular attachment, permanent staining of a fabric, or may influence proteinaceous membrane fouling *etc*.[75] These and many other reasons necessitate the application of physical methods (Table 7.1) in polymer modifications, as explained in the subsequent sections.

7.3.1 PHA Blending and Coating

Polymer surface roughness, chemistry and thermodynamic properties are among the influential features in polymer biocompatibility.[76] Studies have shown that the surface properties of polymers determine the type of molecules that can be adsorbed.[76–78] For example, in tissue engineering, ideal polymeric scaffolds should suitably provide microenvironments for cellular attachment, support, regeneration, proliferation, differentiation and tissue neo-genesis.[79] Cellular attachment and adhesion are suggested to be highly dependent on receptor-mediated interactions that rely on mutual molecular recognition between receptor integrins located on the cellular surface and ligands on the biomaterial surface.[79] Although diverse types of PHA could be used as tissue scaffolding, these materials are reported to lack ligands for biological recognition.[80] Hence, several physical approaches were devised in order to improve the cellular adhesion potential of PHA and its composite (Table 7.1). Blending PHBHHx with PHB resulted in a dramatic increase in the attached L929 cell viability as compared to the neat polymer or PHBHHx digested with lipase or NaOH.[81] On the other hand, coating PHBHHx with hyaluronan smoothed the polymer surface but decreased the contact angle of water to the material surface by approximately 30% resulting in about a 40% reduction in the growth of attached mouse fibroblast (L929) cells when compared with the neat polymer.[77] This kind of coating and blending could be employed in biomaterial design for cellular growth selection.

Similarly, blending the PHA with the anti-fouling agent 4,5-dichloro-2-*n*-octyl-4-isothiazolin-3-one (DCOI) improved the polymer's stability towards

Table 7.1 Typical PHA physical modification methods.

PHA	Modification method	Results/application	Ref.
PHBHHx	Hyaluronan coating	Improved hydrophilicity, low fibroblast L929 cellular growth 1.6×10^5 (4×10^5)[a] cells per ml. Could be employed in biomaterial selection and design.	77
PHBHHx	PHB blending	Improved biocompatibility and higher fibroblast L929 cellular growth 1.9×10^5 (1.8×10^4)[a] cells per cm^2. Could be employed in biomaterial selection and design.	81
mcl-PHA	Crosslinking by γ-irradiation	Higher cross linked density, decreased biodegradability. Increased Young's modulus 129% (114%),[a] improved tensile strength 76% (35%).[a]	82
PHO, PHU	PEGMA grafting by UV irradiation	Reduced blood protein adsorption, platelet adhesion, and improved blood compatibility. Potential application in blood contacting devices.	83,84
PHB	OH^- ion implantation	Improved bioactivity, wettability. Suitable for cell culture scaffold.	85
PHBV	O_2 plasma treatment	Increased hydropilicity, reduces polymer surface roughness, resulting in high cell growth and attachments. A potential scaffold for retinal pigment epithelium cell culture.	86
PHBV	Electrospinning	Improved elastic property, increased wettability. Scaffold for tissue engineering.	87

[a]Numbers in parentheses represent unmodified PHA control values.

environmental degradation and reduced biofouling caused by soil microbes.[88] In fact, blending the PHA with DCOI was observed to postpone the onset of weight loss by up to 100 days, about a 10-fold increase in degradation stability compared to neat PHA film.[88]

Previous studies have shown that PHA granule binding protein (PhaP) and its fusion proteins, such as arginine-glycine-aspartate (RGD) tri-peptide, have higher cell-binding sequences, which improve cell survival, attachment, motility and proliferation, allowing their usage in specific drug delivery devices and cell sorting.[89,90] You *et al.*[89] compared the biocompatibility and cell viability of a scaffold made of neat PHBHHx to that of PHBHHx coated with PHA PhaP fused with RGD (PhaP-RGD). The study showed that the PhaP-RGD coating led to better homogeneity in cell spreading, adhesion, proliferation with improved chondrogenic differentiation and high extracellular matrix production compared with the neat PHBHHx scaffold.

Similarly, PHA repressor protein (PHaR) was reported to contain a hydrophilic DNA binding domain (DBD) and hydrophobic granule binding domain (GBD) allowing it to bind different polymers and biological macromolecules.[91] On the other hand, extracellular matrix proteins such as *Lys-Gln-Ala-Gly-Asp-Val* (KQAGDV) oligo-peptide have been shown to enhance cell adhesion and proliferation.[92] Recently, Dong *et al.*[79] evaluated the cytocompatibility of PHA coated with PhaR and *Lys-Gln-Ala-Gly-Asp-Val* (KQAGDV) polypeptide. Using water contact angle measurements, the coated polymer exhibited a significant improvement in hydrophilicity. Furthermore, in terms of cytocompatibility, the PhaR-KQAGDV coating clearly enhanced the adhesion and proliferation of human vascular smooth muscle cells (HvSMCs) far better than the non-coated controls based on microscopic investigations and CCK-8 assay.[79]

7.3.2 PHA Irradiation

In contrast to other polymer modification methods, irradiation of polymeric materials required no addition of polymer contaminants. Irradiations such as gamma-irradiation normally result in three-dimensional network structures with improved tensile strength. Several studies have demonstrated the cross-linking of unsaturated mcl-PHAs by gamma-irradiation.[82,93,94] The presence of olefinic bonds in PHA side chains provides an avenue for polymer modification by several irradiation processes. A highly cross-linked modified polymer was produced by irradiating unsaturated PHA obtained from tallow-grown *P. resinovorans* with 25–50 kilogray (kGy) of γ-irradiation. The modified polymer showed a reduced degradability, increased Young's modulus and enhanced tensile strength (Table 7.1).[82] The study also showed that radiation treatment up to 50 kGy had no effect on the thermal stability, melting enthalpy (ΔH_m) and storage modulus of the polymer, but induced polymer chain scission.[82] The polymer chain scission was minimized by the addition of linseed oil, which caused a 2.5-fold reduction of ΔH_m in the entire polymer tested. This observation was attributed to the high density of olefinic bonds in linseed oil.[82] A modified cross-linked polymer of PHOU was also prepared by γ-irradiation.[94] Optimal cross-linking was found upon treatment with a 20 kGy dose in an inert atmosphere (N_2). The polymer film was reported to be unaffected upon mild radiation treatment.[94] Smart hydrogels of semi-interpenetrating polymer networks (IPNs) based on PHB and a hydrophilic poly(ethyleneglycol diacrylate) monomers at different compositions were prepared by radiation-induced polymerization using γ-rays within 10–100 kGy.[95] A reduction in polymer thermal melting temperature and crystallinity with increasing radiation dose was observed. This was probably due to the partial destruction of the crystalline region upon exposure to the radiation energy.[96]

Simultaneous irradiation techniques were reported to produce a homogeneously grafted polymer of high purity.[97] The effect of solubilizing solvent on the simultaneous γ-irradiation induced graft copolymerization of

acrylamide onto PHB was also studied.[97] The authors use three different models (eqns (7.1), (7.2) and (7.3)) to describe the percentages of grafting degree (W%), monomer/macromer composition (C_x%) and crystallinity index (CI%), respectively.

$$W\,\% = \frac{m_g - m_i}{m_i} \times 100 \tag{7.1}$$

$$C_x\,\% = \frac{m_x}{m_g} \times 100 \tag{7.2}$$

$$CI\,\% = \frac{A_{\lambda CH_3}}{A_{\lambda C-O-C}} \tag{7.3}$$

where m_g, m_i and m_x are thermogravimetric analysis (TGA) derived graft copolymer weight, neat backbone polymer weight, and weight of the monomer or macromer component under analysis, respectively. $A_{\lambda CH3}$ is the FTIR integral peak area of the band at wavelength (λ) 1382 cm^{-1} assigned to methyl bending vibrations, which is insensitive to the crystallinity changes, and $A_{\lambda C–O–C}$ is the integral peak area at 1185 cm^{-1} corresponding to ester vibrations that is sensitive to changes in crystallinity.[97] In all the solvents evaluated (acetone, methanol, ethanol, ethyl acetate and chloroform), the researchers found that the sample grafted in chloroform had the highest degree of grafting ($\approx$91%) in spite of the chloride radical that was reported to terminate the reaction.[97] In addition, the chloroform-grafted sample was found to exhibit higher water uptake, about 2.5-fold higher than the sample grafted in acetone. This could be due to an increased degree of acrylamide grafting as a result of higher polymer solubilization in chloroform.[97] Several PHA modifications by gamma induced graft copolymerization have been reviewed elsewhere.[98]

Olefinic bonds of unsaturated polymers confer a cross-linking/grafting advantage upon irradiation with ultraviolet (UV) light even in the absence of photosensitizers or initiators.[84] On the other hand, saturated polymers such as PHO normally need photo-initiators (*e.g.* benzophenone) to initiate the cross-linking reaction upon UV irradiation.[83] A series of unsaturated PHAs derived from linseed oil was cross-linked using UV radiation at a wavelength of 300 nm, which resulted in modified PHAs with increased glass transition temperatures and improved curing due to the cross-linking reactions.[99]

For biomedical applications, inhibition of protein adsorption, as well as platelet activation and adhesion on the polymer surface, is critical to the efficiency of the material. For example, use of PHAs as artificial blood contacting devices such as arteries and anticoagulant films was limited by surface-induced thrombosis.[83,100] The adsorption of plasma proteins and adherence of activated platelets onto the polymer surface resulted in their transformation to pseudopods and subsequent release of platelet biochemical content, which in turn activated other platelets leading to the

thrombosis condition.[83] The monoacrylate of polyethylene glycol methacrylate (PEGMA) was grafted with poly(3-hydroxyundecenoate) (PHU)[84] and PHO[83] by UV irradiation of the homogeneous PHA solutions containing PEGMA. In both studies, the surface adsorption tendencies of both graft copolymers towards blood proteins and platelet adhesion were reduced significantly in comparison to the control *i.e.* poly (*L*-lactide) surface. Furthermore, the grafted copolymers exhibited excellent blood compatibility with increasing PEGMA fraction.[83,84] A higher PHA molecular weight (M_w) is associated with a lower degradation rate thus limiting its application as a short-lived drug carrier for molecules such as DNA and functionalized polypeptides. Instead of cross-linking and grafting the PHA, Shangguan *et al.*[101] used UV irradiation to achieve controlled degradation of PHBHHx leading to oligomers bearing reactive radical groups with a quick degradation rate. The authors found the decrement of the M_w to be dependent upon the UV radiation exposure time. The degradation mechanism was extensively reviewed.[9]

7.3.3 Ion Implantation

Ion implantation is another physical method employed in polymer surface modification. Its advantage over other polymer modification methods is that it only modifies the polymer surface layer, without upsetting the bulk polymer's properties.[102] Ion implantation has been successfully applied in several polymer modifications thereby expanding its applications.[102–106] Hou *et al.*[85] successfully implanted hydroxyl ions on the surface of PHB using an electron ion implantation energy of 40 keV when an implantation flux ranging from 1×10^{12} to 1×10^{15} ions per cm^2 was applied. The study reported that the modified PHB showed a better proliferative activity of mouse embryo fibroblast (3T6) cells compared to non-modified PHB. However, it was observed that the ion implantation caused polymer cracks proportional to the intensity of fluence or flux of the hydroxyl ion bombardment.[85] In a similar study, Santos *et al.*[107] improved the hydrophilicity of PHB and PHB-graft-polyvinylacetate copolymers by implanting H^+, Ag^+, and Na^+ ions at different fluences. In both polymers, the treatment resulted in increased hydrophilic characteristics. PHB and PHBHHx wettability was shown to be significantly improved upon implantation with carboxyl ions at an electron implantation energy of 150 keV and ion bombarding fluences ranging from 5×10^{12} to 1×10^{15} ions per cm^2.[102] In comparison to the non-implanted PHA, the polymer surface analysis revealed a decrease in the intensities of $-C-C-$, $-C-O$ and $-C=O$ groups in the COO^- implanted polymer, thus improving its hydrophilicity. Likewise, PHB, PHBV and PHBHHx implanted with C^+ ions revealed improved cytocompatibility when used as supports for culturing mouse embryo fibroblast (3T6) cells.[105] In all polymer samples, it was found that an implantation ion flux of 1×10^{12} ions per cm^2 was the optimal in conferring better cytocompatibility.

7.3.4 Plasma Treatment

When a gas is subjected to extreme heat, or an electric or electromagnetic field applied from a laser or microwave generator, this causes the molecules to ionize thus turning the gas into plasma composed of charged ions, electrons, radicals, and neutral species.[108] When the generated plasma has a uniform thermal equilibrium with its components at the same temperature, such plasma is described as thermal plasma. On the other hand, the plasma is termed a non-thermal having a strongly deflected kinetic equilibrium when excited electrons temperature is higher than the ions and the neutrals.[109] Because of their high temperature, diverse density and complex compositions, plasma interacts with a polymer when it is physically bombarded by excited electrons resulting in polymer surface modification bearing chemical or ionic groups that could participate in further reactions such as cross-linking, grafting, etching, roughening and functionalization. Like ion implantation, plasma treatment has the advantage of modifying the material's surface layer only, without tempering with the polymer's intrinsic mechanical properties.[110] The high discharging efficiency of radio frequency (RF) plasma makes it an excellent choice in this regard.[108]

Despite its excellent biodegradability, compatibility and diverse adjustable mechanical properties,[7] the highly crystalline PHB is strongly hydrophobic, resulting in limited applications due to its slow degradation rate, bioresorbability and poor cell adhesion.[26,28] Surface treatment by plasma is considered as an effective approach to increase the hydrophilicity and wettability of such polymers.[111] Recently, Mirmohammadi *et al.*[108] compared the biocompatibility of the PHB surface upon treatment with O_2 and CO_2 plasma at 50 W discharge for 3 min, and found that O_2 plasma treated PHB showed much improvement. The results indicated that neat PHB with an irregular and coralloid surface was modified to a more regular surface morphology with improved roughness (nano-protrusion and nano-indentation) upon plasma treatment. This modification resulted in enhanced cell–polymer electrostatic interactions, and improved growth of attached L929 fibroblasts. Based on FTIR-ATR analysis, it was observed that the surfaces of plasma treated PHB were functionalized *via* endowment of oxygen functional groups such as –OH, COO– and –CO– that improved the wettability of the treated surfaces.[108] Hasirci *et al.*[112] studied the influence of oxygen RF-plasma treatment on the surface and bulk properties of PHBV. Their findings showed that the plasma-treated films absorbed more water than the untreated films, and the degree of absorption depends on the applied plasma discharge power. They further observed a decrease in water contact angles and an increase in oxygen–carbon atomic ratio upon treatment, indicating improved hydrophilicity due to an increase in the oxygen-containing functional groups on the surface of the polymer.[112]

Radio frequency glow discharge (RFGD) plasma generated at 100 kHz was used to modify a PHO surface that was subsequently grafted with acrylamide in aqueous solution.[110] The amount of grafted amide and wettability were

found to increase proportionally to the increasing plasma discharge power. The modified polymer biocompatibility was evaluated using Chinese hamster ovary cells where surfaces treated with 30 W of plasma have moderate hydrophilicity while showing excellent cell adhesion and high growth. Improved hydrophilic properties and highly stable modified PHB-amino groups were obtained when low pressure microwave ammonia plasma treatment of the polymer surface was used.[113] The presence of the amino group on the PHB surface was established using X-ray photoelectron spectroscopic (XPS) analysis after the treated sample was chemically labeled with 4-trifluoromethyl benzaldehyde.

As previously mentioned, the blood coagulation process (thrombosis) is a complex process that involves the participation of blood proteins and platelets. Protein adsorption onto polymeric material induced platelet adhesion leading to thrombosis. Thus, an effective anticoagulant film is expected to have high protein resistance characteristics. Increased surface wettability, charge, morphology and functional groups are among the factors indicated to help inhibit protein adsorption onto a polymer's surface.[114,115] The wettability and protein resistance characteristics of P3HB4HB were improved upon treatment with oxygen plasma for 10 minutes.[116] The study showed that the platelet resistance characteristic of the polymer was improved by 96.8%; likewise, the protein (BSA) resistance characteristic was also improved by 27% and 57.5% in phosphate buffer and aqueous solutions, respectively.

It has been reported that an effective cure for retinal disorder due to retinal pigment epithelium (RPE) degeneration is currently unavailable.[86] Oxygen plasma treatment was used to improve the hydrophilicity of a scaffold based on PHBV containing 8 mol% hydroxyvaleric acid, which was used to successfully culture RPE (D407) cell lines. An engineered scaffold with a rougher surface and enhanced hydrophilicity for cardiovascular tissue was prepared from PHBHHx-coated silk fibroins modified using low temperature atmospheric plasma.[117] Similarly, ammonia plasma treated PHBHHx followed by fibronectin coating encouraged better growth of human umbilical vein endothelial cells (HUVECs) and rabbit aorta smooth muscle cells (SMCs) on its surface compared to the untreated polymer, and could serve as a potential material for the luminal surface of vascular grafts.[118] A plasma-induced polymerization technique using polyethylene glycol (PEG) and ethylenediamine (EDA) as a co-monomer was used to modify the surface of an electrospun PHB nanofibrous scaffold resulting in a material with enhanced cellular proliferative activity.[119] Similarly, the same plasma-induced polymerization was used to graft polyacrylic acid onto PHAs of different monomeric compositions, resulting in modified PHAs with relatively unchanged mechanical properties but enhanced hydrophilic properties.[120]

7.3.5 Electrospinning

Electrospinning is a method that employs the use of a high electrical voltage to produce polymer fibers of different diameters from polymer

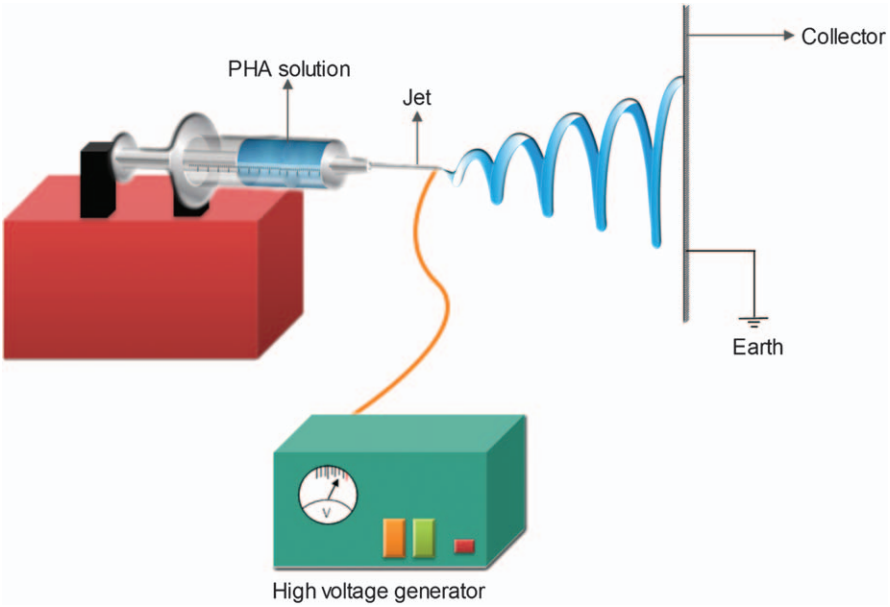

Figure 7.11 PHA electrospinning set-up.

solutions (Figure 7.11).[121,122] Polymer electrospinning is used to produce a polymeric scaffold that mimics natural extracellular matrices. The solution's viscoelasticity, its surface tension and jet charge density were reported to be among the influential factors in polymer electrospinning processes.[122] Jet charge density is affected by the applied electrostatic field and solution conductivity. For example, Fong *et al.*[122] reported that addition of sodium chloride to an aqueous solution of polyethylene oxide increased the net charge density of the spinning jet. In the case of a low molecular weight polymer solution, the researchers reported that the electrically driven spinning of the solution resulted in polymer droplets (electrospray) due to the capillary breakup of the spinning jet as a result of surface tension.

Kwon *et al.*[87] used a high voltage (10–20 kV) to produce ultrafine electrospun PHBV fiber mats that were used as scaffolds for tissue engineering. The researchers observed that the electrospun nanofiber's diameter decreased with the decreasing concentration of polymer solution and increasing applied voltage. In comparison to the neat polymer, they found that the electrospun PHBV exhibited about a six-fold increase in elongation to break than the PHBV cast film. The study showed that due to the increased porosity and surface area of the electrospun PHBV fibrous mat, seeded chondrocytes adhered to and grew better on it than on the cast PHBV film. A similar study on the improved biocompatibility of electrospun PHBV was reported.[123] When studying the cell attachment efficiency of the electrospun nanofibers by seeding chondrocytes derived from rabbit ears on both the PHBV cast film and electrospun PHBV nanofibrous mat, the researchers observed about a

two-fold increase in the chondrocytes' attachment after two hours of incubation when electrospun nanofiber was used as compared to the cast film.[123] Yu *et al.*[121] observed a decrease in the crystallinity and crystallization rate by doping zinc oxide (ZnO)/PHBV electrospun nanofiber with ZnO nanoparticles. They attributed the observed effect to the interaction between the hydroxyl groups on the ZnO nanoparticles' surface and the polymeric carboxylic groups. Ying *et al.*[124] evaluated the biocompatibility and biosorption characteristics of the electrospun scaffold of P3HB4HB through subcutaneous implantation of the fibers in rats. The researchers found a highly increased tissue response with increasing content of 4HB monomer.

7.4 Modification of PHAs with Enzymes

PHA modification *via* an enzyme-mediated process is seen as a mild, specific and environmentally-friendly method. In this section, PHA modification using enzymatic degradation and/or synthesis methods in both *in vivo* and *in vitro* processes is discussed. Also included in the discussion is enzymatic modification of PHA using the degradation products of PHA itself.

7.4.1 *In Vivo* Enzymatic Degradation of PHA

During the bacterial fermentation process, PHA granules are accumulated inside the cells in the presence of excess carbon source(s) but limited essential nutrients such as nitrogen, oxygen, phosphorous, potassium, or sulfur.[125] In a reverse situation where the microbes are starved of a carbon source in the presence of abundant nutrients, they will start producing intracellular PHA depolymerase and dimer hydrolase to degrade the accumulated PHA and continue growing.[126] The monomer produced from the intracellular degradation is subsequently oxidized by the cell to form acetoacetate. In an attempt to bypass the accumulated PHA hydrolysis, Lee *et al.*[127] proposed a process that incorporates continuous limitation of the carbon source and nutrient(s) in an anaerobic condition. The absence of oxygen inhibits the cells from metabolizing the 3HB monomers from intracellular PHB degradation by lowering the concentration of *R*-3-hydroxybutyrate dehydrogenase, which in turn minimizes the conversion of 3HB to acetoacetate.

It has been reported that a high yield of 3HB (96%) can be obtained within a relatively short time (30 min) in *Alcaligenes latus* by using a fed-batch culture system with sucrose as a carbon source. The cells with stored PHB were collected and incubated at pH 4 and 37 °C to provide an environmental condition in which cells exhibit high activity of intracellular PHA depolymerase and low activity of (*R*)-(–)-3-hydroxybutyric acid dehydrogenase.[127] Other identified factors that contribute in *in vivo* depolymerization are substrate concentration and extracellular pH. Ren *et al.*[128] reported that the optimal initial pH range for initiation of intracellular depolymerization of PHA by *Pseudomonas putida* was 8–11, and pH 11 after commencing

monomer release.[128] A drop in the solution's pH was due to secretion of PHA monomers by the bacteria.[12]

7.4.2 *In Vitro* Enzymatic Depolymerization

7.4.2.1 Extracellular PHA Depolymerase

Many PHA-degrading microorganisms have been isolated and their ability to secrete extracellular PHA depolymerase could be exploited. The degraded monomer can be extracted and used as a macro-initiator for further modification.[129] The extracellular PHA depolymerases for mcl-PHA have been identified mainly from Gram-negative bacteria, predominantly *Pseudomonas* species; *Pseudomonas fluorescens* GK13, actinomycete species; *Streptomyces roseplus* SL3, and scl-PHA depolymerase from *Alcaligenes faecalis* T1.[130–132] This enzyme belongs to the family of serine hydrolases with lipase consensus sequence *Gly-X-Ser-X-Gly* and it is strongly hydrophobic.[133] The catalytic triad for PHA depolymerase consists of a serine residue that acts as a nucleophile with aspartate (or glutamate) and histidine to stabilize it (serine-histidine-aspartate).[134,135]

Shirakura *et al.*[131] observed that PHB depolymerase acts as an endo-type hydrolase as the enzyme only cleaves at the second ester linkage from the hydroxy terminus of the trimer, tetramer, pentamer and higher oligomers as shown in Figure 7.12. At the same time, the PHA depolymerase prefers PHA with alkyl side chains rather than linear PHA.[135] According to Mukai *et al.*,[136] PHA depolymerase enzyme is very selective towards chiral monomers, especially alkyl side chains such as 3HB rather than 4HB. The rate of enzymatic hydrolysis is primarily dependent on the composition and length of the PHA side chain. Longer side chains provide steric hindrance for the enzyme to be adsorbed effectively on the polymer backbone chain as shown in Figure 7.13.[137]

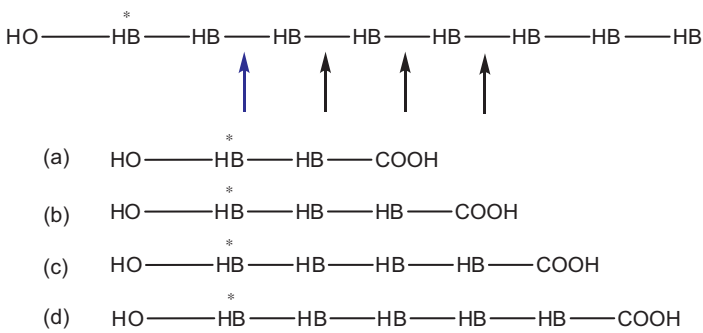

Figure 7.12 Bond cleavage during initial action of poly(3-hydroxybutyrate) depolymerase on radioactive oligomers start from blue arrow. (a), (b), (c), (d) are some products of hydrolysis with (a) degradation product not undergoing further hydrolysis.

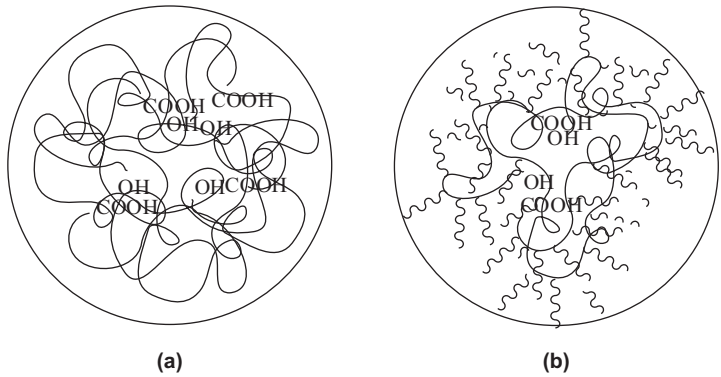

Figure 7.13 Schematic folding structure of (a) linear PHA, (b) branched PHA.

Several key factors in enzymatic catalysis include their activity, stability, substrate concentration and temperature. Optimization of these factors will steer the enzyme towards the desired activity. Media pH was shown to affect the ionizable groups at the active site of the enzyme.[138] Gangoiti *et al.*[139] reported that P(3HO) depolymerase showed its maximum activity at pH 9.5 and a temperature of 40 °C.

7.4.2.2 Lipases

Lipases are subclass of esterases, capable of hydrolyzing esters, fats and lipids in aqueous media, hence they are described as hydrolases belonging to class EC 3.1.1.3. Lipases are one of the most versatile enzymes because they can catalyze many different reactions such as esterification, interesterification, hydrolysis, alcoholysis, peroxidation, aminolysis, and epoxidation.[140,141] Most lipases have similarities in their amino acid sequence including within the catalytic region, ***His****-X-Y-Gly-Z-****Ser****-W-Gly* or *Y-Gly-****His****-****Ser****-W-Gly* where *W, X, Y and Z* refer to unspecified amino acid residues.[142] These enzymes are stereospecific towards ester bonds thereby eliminating any undesirable by-products of the reaction.[143] Lipase has been used extensively as a biocatalyst in industry because of its high thermal stability, versatile pH range and it can be used repeatedly if immobilized (*e.g.* Novozyme 435).[144]

In PHA modification, Mukai *et al.*[136] reported that lipases originated from eukaryotes have broad specificities with the abilities to erode P(3HP), P(4HB), P(5HV) and P(6HH) films compared to prokaryote lipases, which could barely degrade all the polymers except P(3HP). This shows that lipases from prokaryotes have high substrate specificities for the hydrolysis of PHA. Jaeger *et al.*[135] indicated that *Pseudomonas alcaligenes*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Burkholderia lemoignei* and *Bacillus subtilis* lipases show specificities towards ω-hydroxyalkanoic acid over α-hydroxyalkanoic acid. The absence of alkyl side chains in the polymer backbone

allows for more flexibility and hydrophilicity of the aliphatic main polymer chain thus resulting in better engagement between the polyester chain and the active site of lipases.[136,145,146] For lipase catalyzed degradation and esterification reactions, different factors require special attention. In order to optimize lipase catalyzed esterification activity in organic solvents, the temperature of the reaction plays an important role in enzyme stability and the solubility of substrates such as sugar moieties and alcohol since the major problem with hydrophilic moieties is that they are insoluble in organic solvents.[147–149] It has been reported that at a temperature of 50 °C, a high yield of poly(1′-*O*-3-hydroxyacyl-sucrose) formed using Novozyme 435 was shown to be due to an improvement in substrate dissolution.[28] While for lipase catalyzed PHA degradation in toluene, the reaction rate was almost the same between 40 and 60 °C.[150] However, factors such as type of lipase,[135] concentration[146] and degree of initial crystallinity[151] were reported to influence the rate of PHA degradation by lipases.[151]

7.4.3 Degradation Products

7.4.3.1 Chiral Monomer of PHA

Recently, there has been a surge in demand for pure biodegradable PHA enantiomers due to their reported biological activities.[152] The extensive use of pure enantiomers in industries such as medicine, agriculture and the food industry boosts the need for its large scale production.

(*R*)-(–)-Hydroxycarboxylic acid can be widely used as a chiral building block for the synthesis of fine chemicals such as antibiotics, vitamins, aromatics, and pheromones.[153] Its hydroxyl and carboxylic groups are amendable to modification in addition to being utilized as precursors for the synthesis of new compounds.

In another study, PHA synthase from *R. eutropha* was used to polymerize PHB and PHV on hydrophobic highly oriented pyrolytic graphite (HOPG) and alkanethiol self-assembled monolayer (SAM) surfaces, which are used to support other functional biomolecules such as streptavidin and biotin. The surface modification was reported to have biomedical and biotechnological applications.[154]

7.4.3.2 PHA Oligomers

There has been a great deal of interest in PHA oligomers because of their *in vivo* biodegradability and bioresorbability. Furthermore, Tasaki *et al.*[155] stated that dimers and trimers of 3HB can be rapidly converted to monomers in rat and human tissues. Thus, various kinds of dendrimers made up of oligo-HA could be developed and used for various drug delivery applications.[152] Oligomers from the degradation of neat PHA can be used for grafting copolymers at a carboxylic end terminus. Oligomers show a narrower molecular weight distribution compared with neat PHA and they come

with relatively higher functionality. Grafting oligomers allows more controlled processes and a high yield (83%).[156]

7.4.4 Surface Modification

PHAs' excellent mechanical properties, biocompatibility and degradability have made them useful in the field of tissue engineering. These polymers could prove very useful as tissue scaffolds for implantation purposes.[157] However, the smooth surface of a solvent-cast PHA scaffold is a major obstacle for cell attachment in tissue regeneration processes.[158] This warrants the enzymatic catalyzed surface erosion of PHA. Moreover, the PHA surface lacks a bioactive ligand to couple with a bioactive molecule in targeting devices or biosensors. Hence, PHA surface erosion or roughening is needed to provide a corrugated trough to immobilize bioactive molecules such as insulin,[159] fibronectin[160] and collagen[161] while enhancing its cell attachment or cell proliferation characteristics and thus expanding its biomedical applications.[162] Ihssen *et al.*[163] used extracellular PHA depolymerase from *Pseudomonas fluorescens* as the capture ligand to immobilize a fusion protein on to mcl-PHA microbeads of 200–300 nm size (Figure 7.14). This arrangement could be employed as a probe for targeting proteins in drug delivery, protein microarrays, and protein purification. Furthermore, the researchers reported that the binding capacity was comparable to similar-sized polystyrene particles commonly used for antibody immobilization in clinical diagnostics.[163]

Recently, the use of nanoparticles as drug delivery systems for targeted release at specific parts of the body represents a promising solution for cancer treatment.[164] Surface functionalization of PHA nanoparticles is needed to improve targeting efficiency in delivering drug molecules to target cells. This modification can be done *via* PHA-protein block copolymerization. Generally, during *in vivo* synthesis of PHA, PHA synthase is a key enzyme that catalyzes the polymerization of hydroxyacyl-Coenzyme A, a

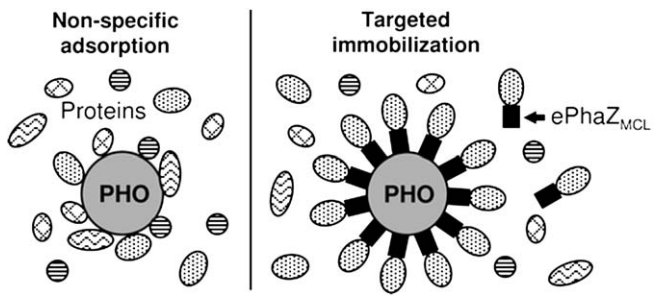

Figure 7.14 Immobilization of targeted protein onto PHA using extracellular PHA depolymerase, reprinted from Ihssen *et al.*[163] with permission from the American Chemical Society.

substrate for PHA biosynthesis. On the other hand, during *in vitro* PHA synthesis, PHA synthase and the hydrophobic chain of the polymer were covalently bound resulting in the formation of an amphiphilic block copolymer in which PHA synthase can further be modified through protein engineering to improve the protein-PHA copolymer.[165,166] It has been reported that the first 100 N-terminal amino acid residues of PHA synthase can be removed without affecting the enzyme's activity.[167] Thus, additional ligand fusion can still be carried out without affecting the protein's native catalytic activity. Incorporating a tumor specific ligand, RGD4C, with PHA synthase helps PHA nanoparticles adhere more effectively on MDA-MB 231 breast cancer cells.[168] Modifications of the PHA nanoparticles were described by Kim *et al.*[165] where the use of PHA synthase from *Ralstonia eutropha* H16 for the formation of protein-PHB copolymer micelles (involving polymerization and self-assembly of 3-hydroxybutyryl-CoA (3HB-CoA)) in aqueous solution at room temperature resulted in a hydrated shell. Concomitantly, hydrophobic drug molecules are integrated into the core of the shell. On the other hand, Lee *et al.*[168] proposed a coupling between PHB emulsion and enzymatic protein functionalization. Then growing PHB tail chain arising from the action of PHA synthase that was fused earlier with RGD4C and this arrangement was able to form hydrophobic interactions with the surface of the PHB nanoparticle emulsion containing drug molecules. As the tail chains grew continuously, the surface of the nanoparticles was covered with RGD4C peptide-PHB copolymer that targets breast cancer cells. Paik *et al.*[166] showed that PHB nanoparticles can also bind to a solid surface. In their study, PHA synthase was fused to a *His*-tag (10x-histidine) expressed in a recombinant *E. coli* synthesizing PHB to produce a protein–polymer hybrid with *His*-tag end-functionality. The *His*-tag bound tightly with a Ni^{2+}-nitrilotriacetic acid (Ni-NTA) derivatized solid surface (silicone or agarose). All these modification methods are excellent demonstrations of synthesizing a wide variety of protein functionalized PHAs with novel properties.

PHA surface erosion is normally initiated at micro-holes on the PHA's surface that allow enzymes and water molecules to adhere to the film surface, commencing the hydrolytic process.[169,170] At the beginning, water molecules enter the amorphous regions within the film, which triggers the enzyme-catalyzed hydrolysis of the ester bond.[171] During the hydrolysis process, the roughness of the PHA film increases over time.[172] While the enzyme is thought to attack mainly the amorphous region, wide angle X-ray diffractography revealed a decline in the crystalline peak after 22 hours of reaction, which indicated that the crystalline region was also hydrolyzed by lipase after the amorphous region was eroded.[172] Selective surface erosion using PHB depolymerase helps to liberate the 3HB monomers from the surface of P(3HB-*co*-4HB) and leave the undegradable 4HB monomers on the polymer surface.[173] Different polymer constitutions on the polymer surface may exhibit different degradation properties. Among the parameters that contribute to the enzymatic degradation of PHA films are the molecular

weight of the polymer, crystallinity, monomer composition, porosity and roughness of the polymer surface.[173] In order to measure the rate of degradation, changes in the weight loss of the PHA film over time can be monitored. While the biopolymer chains could be completely degraded into monomers and dimers of 3-hydroxyalkanoic acids by PHA depolymerase,[174] this process is very slow. Williams *et al.*[175] measured the molecular weights at the surface *versus* the interior of the PHO implants but no significant differences were detected. This showed that homogeneous hydrolytic breakdown of the polymer occurred inside the mice.[176]

7.4.5 PHA Functionalization

Previously, applications of lipase had been limited because the enzyme was thought to work effectively only in environments with a high water content, and would rapidly lose its activity in organic solvents.[177] Later, the enzyme was found to remain catalytically active in organic solvents, and this generated huge interest among researchers and industries as most of the industrial substrates are hydrophobic in nature. Utilization of an organic solvent as a reaction medium helps to improve hydrophobic substrate solubility and simultaneously elevates the rate of the reaction. In addition, a micro-aqueous environment enables reactions that are impossible in water, such as esterification, to be carried out.[178,179] It has been suggested that organic solvents may play a role in prolonging enzyme activity by replacing the molecules of water on the enzyme with solvent molecules.[177] Reactions in water usually caused downstream difficulties in the separation of soluble enzyme and products. However, in organic solvent media, the enzyme is insoluble and this facilitates product recovery.[180]

The functionalization of polymers may involve chemo-enzymatic synthetic steps. This can be seen in the esterification of poly (ε-caprolactone) with hydrophilic moieties in the presence of CAL B as a biocatalyst where tetrahydrofuran, dichloromethane and dioxane were used as reaction media.[149,181] Longer chains of PHA pose a steric hindrance for the enzyme to bind effectively with the polymer substrate at the carboxyl end terminus for transesterification. To overcome this limitation, enzyme catalyzed hydrolysis of the poly (ε-caprolactone) was carried out prior to chemical transesterification.[181] The enzymatic step not only facilitates the subsequent step but also helps to generate more carboxyl end terminals for transesterification.[81] In another study, Gumel *et al.*[182] studied the use of two organic solvents *viz.* dimethylsulfoxide (DMSO) and chloroform at a 1 : 4 ratio to dissolve reaction substrates with contrasting solubilities *i.e.* sucrose and PHA. Since a small amount of DMSO was sufficient to dissolve enough sucrose, and then mix with PHA in chloroform, a system that behaves largely like a single-phase system could be achieved. Consequently, the effects of interfacial resistance can be avoided. In this system, the researchers demonstrated a successful functionalization of a medium-chain-length PHA with sucrose thereby improving the modified polymer film's biodegradability.

Ravenelle and Marchessault[183] reported the transesterification of PHB with monomethoxy poly (ethylene glycol), mPEG using a bis(2-ethylhexanoate) tin catalyst at 190 °C to form diblock copolymers. However, they were unable to control the molecular weight of the PHB due to thermal degradation that occurs during the high temperature reaction. Gumel *et al.*[182] demonstrated the use of Novozyme 435 for the transesterification of PHA and sucrose in an organic solvent mixture at a mild temperature of 50 °C. From the GPC analysis, the polydispersity index showed a low value at 0.7 with a higher number average molecular weight (M_n) compared with a low weight average molecular weight (M_w). This indicated that less variation in the average molecular weight could be achieved using an enzyme-mediated transesterification process. The scheme of the transesterification process is shown in Figure 7.15.

In another study, Novozyme 435 was used to produce a novel PHB block copolymer based on PHB-PCL in microaqueous media.[184] Such block copolymers were reported to have expanded biomedical applications due to their excellent thermoplastic properties.[184]

The main factor affecting enzyme activity is the molecular water layer on the surface of the enzyme. The major causes of low activity are reduced conformational stability, uncontrolled pH and unfavorable substrate desolvation.[177] Since the amount of water retained naturally by the enzymes becomes the main factor affecting enzyme stability, solvent hydrophobicity (log *P*) value is a convenient indicator of solvent suitability as a reaction medium for the enzyme's activity.[185] While there is great concern over the accumulation of the water by-product that reverses the catalysis direction in lipase catalyzed esterification reactions, it has no relevance to other systems that utilize enzymes such as PHA synthase. For example, two new functionalized PHAs containing cyclopropane and chlorine *viz.* 3-hydroxy-3-cyclopropylpropionate (3CyP3HP) and 3-hydroxy-4-chlorobutyrate (4Cl3HB), respectively, were enzymatically produced using PHA synthase from *Escherichia shaposhnikovii* in an aqueous solution.[186]

At room temperature, PHA is soluble in chlorinated and other organic solvents such as dichloromethane, chloroform, carbon tetrachloride, dichloroethane, chloropropane, tetrahydrofuran, 1,2-propylene carbonate, toluene and hot acetone.[187] In lipase-catalyzed modification of PHAs, the benefits of using hydrophobic organic solvents over an aqueous system include increased solubility of nonpolar substrates, the enzyme favoring the ester-bond synthesis rather than hydrolysis, and elimination of microbial growth that usually contaminates the aqueous reaction mixture. Several solvent systems have been studied such as a water-and-hydrophilic solvent (monophasic), hydrophobic organic solvent (monophasic), water-and-water immiscible (two phase) solvent, and a nearly dry organic solvent system.[188] The advantage of a monophasic system is the minimal diffusion resistance between the substrate in the hydrophilic solvent and the water phase, and the maximum dissolution of substrate concentration that leads to an increase in the reaction rate.[188] In crystalline PHA, the polymer backbone

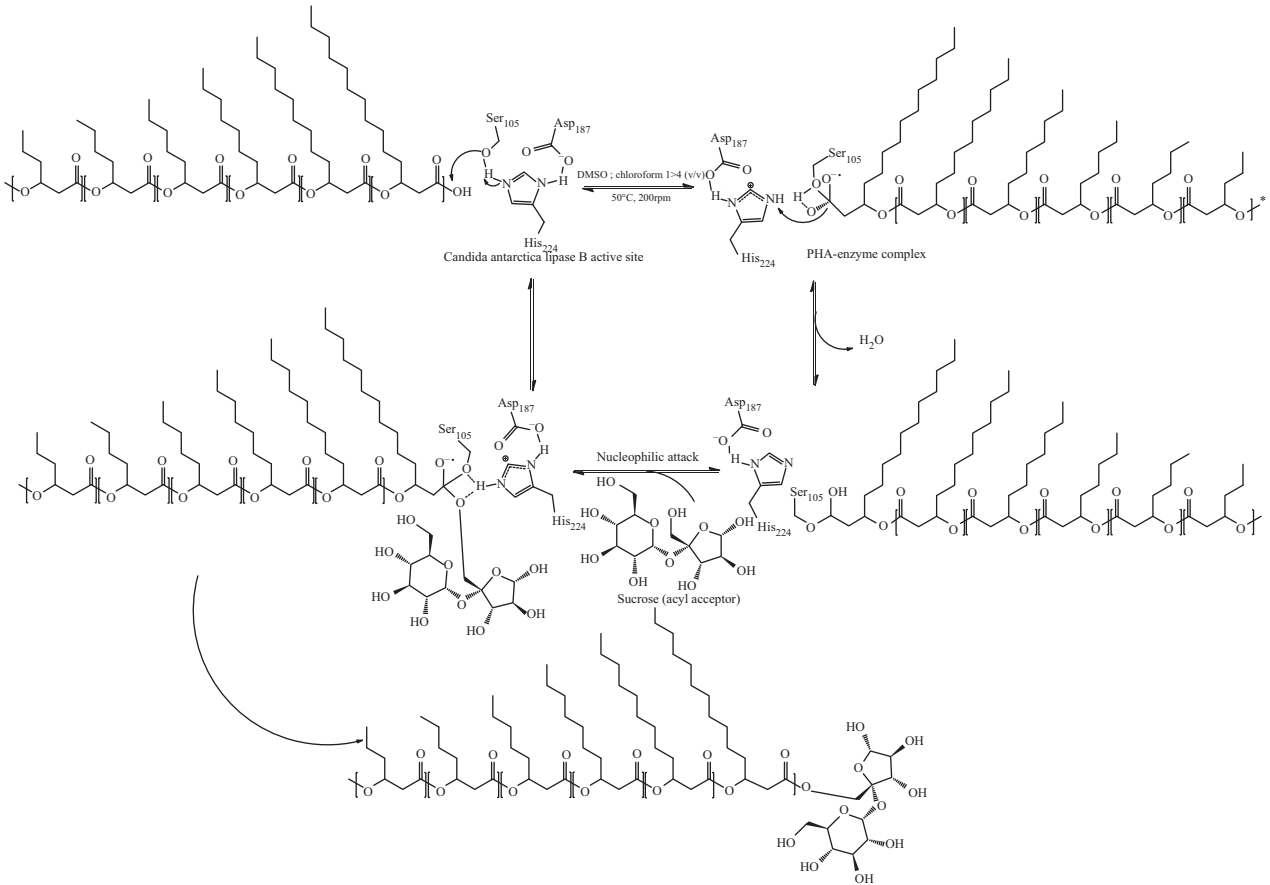

Figure 7.15 Scheme of transesterification of medium-chain-length PHA with sucrose using *C. antarctica* lipase B in an organic solvent mixture.[182]

chains were clumped together, leaving the alkyl side chains pointing outward, covering the polymer surface. When the polymers were dissolved in a solvent, the solvent molecules diffused through the polymer matrix until they reached the polymer core and stretched the polymer backbones to form a swollen, solvated mass. Then, it broke up and the polymer chains started to disperse into true solution. At this moment, the polymer backbones and both functional groups were exposed for enzyme attack.[189] Direct contact of enzyme with organic solvent results in the preferential partitioning of the enzyme's hydrophobic part towards the solvent with simultaneous changes in enzyme conformation, and this may eventually lead to enzyme deactivation.[190] When a two-phase system is introduced to minimize the inactivation of enzyme molecules, denaturing at the interphase between water and the organic phase may still occur albeit at a lower frequency.[188]

7.5 Conclusions

The demand for smart biodegradable PHAs with the flexibility to incorporate specific traits that extend their niche applications, and the difficulty encountered in their *in vivo* production by conventional biosynthesis, have brought about the current interest in neat polymer modification and functionalization *via* chemical, physical and enzymatic processes. The success of these processes largely depends on the extent to which the modification confers the desired trait(s) to the polymer allowing it to perform the targeted function efficiently. Depending on the type of PHA to be modified and its intended end uses, optimal modification calls for sensible manipulation of the process conditions such as catalyst loading, contact or exposure time, reactant or sample concentration, treatment dose *etc*. With rational and proper choice of modification strategies, a highly hydrophobic and crystalline PHA could be modified into a unique polymer with desired bioactive properties, a marked increase in wettability, elasticity and storage modulus characteristics, thereby allowing the functionalized polymers to be used in diverse novel applications.

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