# 1

# Polyesters

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## 1.1

## Historical Background

### 1.1.1

### Biomedical Applications

Biomaterials are defined as any materials intended to interface with biological systems to analyze, treat, or replace any tissue, organ, or function of the body [1]. The current trend in biomaterial development is shifted toward the use of biodegradable materials that have definite advantages in the fields of tissue engineering [2] and drug delivery [3]. The general principle is to use a material that achieves a specific therapeutic task and is subsequently, over time, degraded and removed harmlessly from the body. As an increasingly relevant part of the medical device and controlled release industry, biodegradable polymers are used to fabricate temporary scaffolds for tissue regeneration, medical sutures, and nano- or micro-scale drug delivery vehicles [4–6].

The important properties that are required for biodegradable biomaterials can be summarized as follows:

- Nontoxic and endotoxin-free, aiming to minimalize unwanted foreign body responses upon implantation.
- Degradation time should be matched to the regeneration or required therapy time.
- Mechanical properties must be suited to the required task.
- Degradation products should be nontoxic and readily cleared from the body.
- Material must be easily processed to allow tailoring for the required task.

Although natural polymers such as collagen have been used in medical applications throughout history, synthetic polymers are valuable also, as they allow us to tailor properties such as mechanical strength and erosion behavior. Naturally

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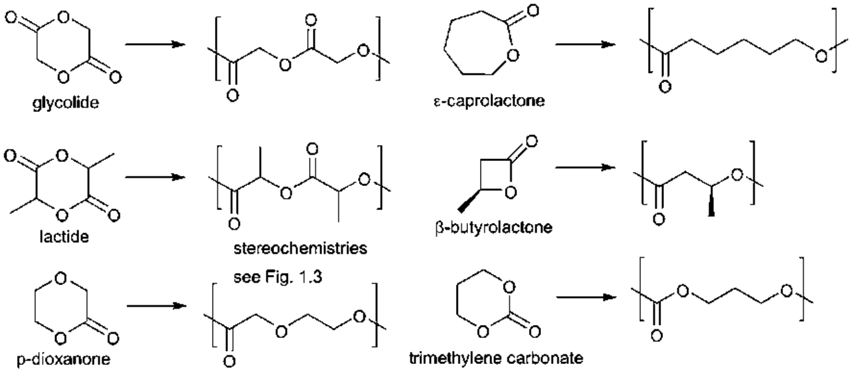
occurring biopolymers are typically degraded by enzymatic means at a rate that may be difficult to predict clinically. Furthermore, natural polymers may have unwanted side effects arising from inherent biological activity. This has led to the widespread use of biodegradable synthetic polymers in therapeutic applications. Of this class, biodegradable aliphatic polyesters, which are degraded hydrolytically, are by far the most employed.

### 1.1.2
### Poly(Hydroxycarboxylic Acids)

All polyesters are, in principle, hydrolytically degradable. However, only (co)polyesters with short aliphatic chains between ester bonds typically degrade over the time frame required for biomedical applications. The major group of this material are the poly(hydroxycarboxylic acids), which are prepared via ring-opening polymerization of lactones or cyclic diesters. Indeed, the first biodegradable polyester used as a medical suture in the 1960s was based on the polyglycolide. Scheme 1.1 shows the most common monomers and the polymers they produce. These can be summarized as diglycolide, stereogenic dilactides, lactones such as ε-caprolactone and stereogenic β-butyrolactone, the cyclic trimethylene carbonate, and *p*-dioxanone. As the polymerization methods of these monomers are broadly applicable to each, copolymers such as poly(lactide-*co*-glycolide) are readily produced.

Another source of poly(hydroxycarboxylic acids) is from bacteria, which store polyesters as their energy source [7]. These polymers are known as polyhydroxyalkanoates (PHAs) in the literature. The most common polymer derived from bacteria is poly(3-hydroxybutyrate), which has the same structure as the polymer which can be obtained from optically active β-butyrolactone [8]. Poly(3-hydroxybutyrate) formed in this way is strictly stereoregular, showing the (*R*) configuration. Biotechnologically produced polymers are discussed in more details in Chapter 2 of this handbook.



**Scheme 1.1** Common cyclic monomers for the preparation of polyester derivatives.

## 1.2
## Preparative Methods

### 1.2.1
### Poly(Hydroxycarboxylic Acid) Syntheses

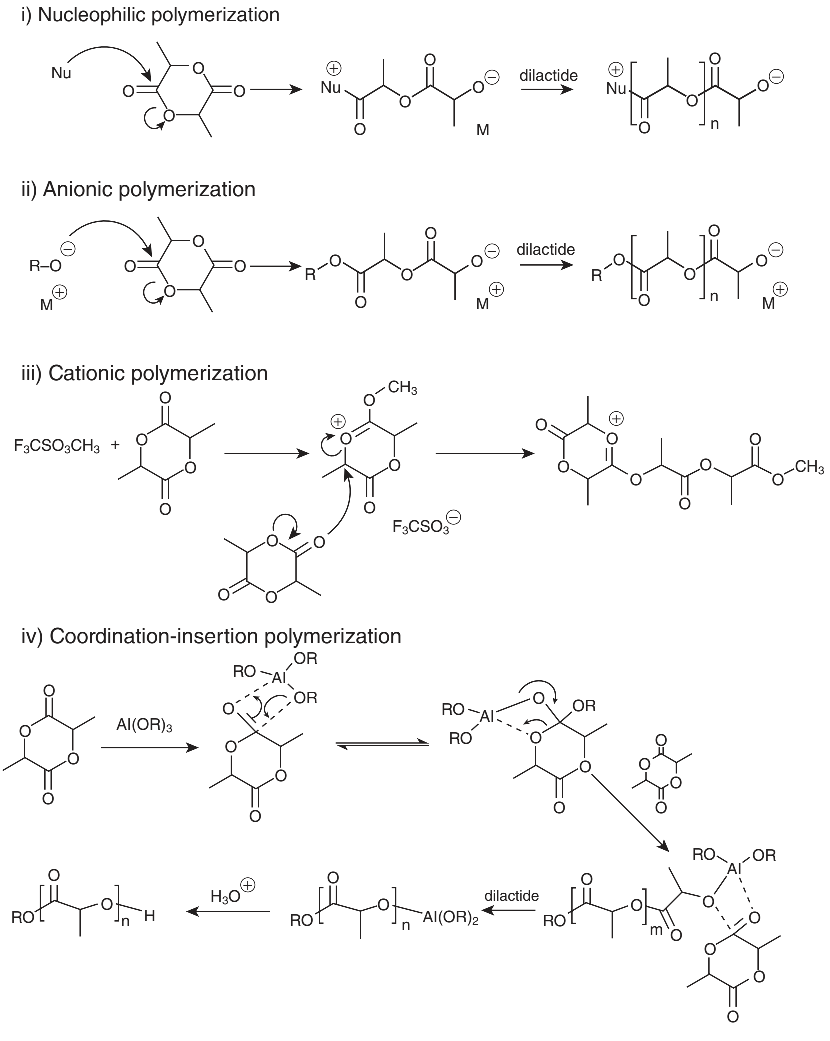
Polyesters can be synthesized via the direct condensation of alcohols and acids. This may take the form of condensing dialcohols and diacids, for example, AA + BB systems, or the direct condensation of hydroxycaboxylic acid monomers, for example, AB systems. Various catalysts and coupling reagents may be used but typically the polyesters formed in this manner have low and uncontrolled molecular weight and are not suitable for biomedical applications. The majority of cases where a high degree of polymerization was obtained came via ring-opening polymerizations of cyclic monomers of the type shown in Scheme 1.1 [9]. The cyclic dilactones are prepared from the corresponding hydroxycarboxylic acid by elimination of water in the presence of antimony catalysts such as $Sb_2O_3$ [10]. These dimers have to be purified rigorously if high degrees of polymerization are sought, as impurities such as water and residual hydroxycarboxylic acids can hinder polymerization. Enantiomerically pure lactic acids are typically produced by fermentation.

Ring-opening polymerizations may be initiated by nucleophiles, anionically, cationically, or in the presence of coordinative catalysts. Representative mechanisms are shown in Scheme 1.2. However, precise mechanisms may vary from case to case and are an ongoing important area of study [11, 12]. As a testament to the popularity of the ring-opening polymerization approach, over 100 catalysts were identified for the preparation of polylactide [13].

The typical complex used for the industrial preparation of polyglycolide derivatives is tin(II)-bis-(2-ethylhexanoate), also termed tin(II)octanoate. It is commercially available, easy to handle, and soluble in common organic solvents and in melt monomers. High molecular weight polymers up to $10^6$ Da and with narrow polydispersities are obtained in a few hours in bulk at 140–220 °C. Approximately 0.02–0.05 wt% of catalyst is required. Care must be taken when polymerizing dilactides, if stereochemistry is to be preserved. This means that milder conditions are to be selected relative to the homopolymerization of diglycolide.

For the copolymerization of dilactide and diglycolide catalyzed with tin(II) octoate, different reactivities are observed. A chain with a growing glycolide end will add a further diglycolide with a preference of 3:1. With a terminal lactide unit, the preference for diglycolide is 5:1. Due to this, glycolide blocks tend to form, separated by single dilactides. One possibility to improve the homogeneity of the composition of the obtained polyesters is the online control of the monomer ratio by addition of further monomer. However, this method is technically complicated.

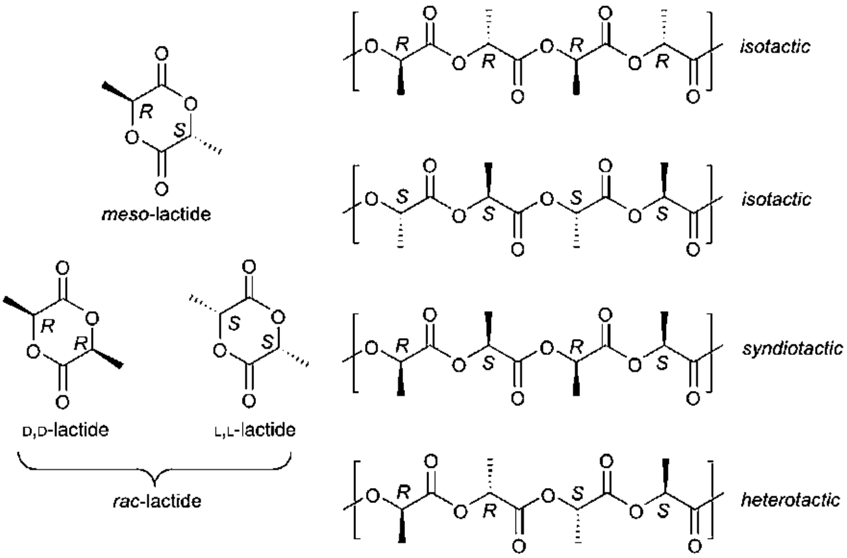
The mechanism is a nonionic coordinated insertion mechanism, which is less prone to the side reactions commonly found in ionic polymerizations, such as transesterification or racemization [14, 15]. It has been found that the addition of alcohols to the reaction mixture increases the efficiency of the tin catalyst albeit



**Scheme 1.2** Overview of various mechanisms relevant to polylactide synthesis.

by a disputed mechanism [16]. Although tin(II)octoate has been accepted as a food additive by the U.S. FDA, there are still concerns of using tin catalysts in biomedical applications.

Aluminum alkoxides have been investigated as replacement catalysts. The most commonly used is aluminum isopropoxide, which has been largely used for mechanistic studies [17]. However, these are significantly less active than tin catalysts requiring prolonged reaction times (several hours to days) and affording polymers with molecular weights generally below $10^5$ Da. There are also suspected links between aluminum ions and Alzheimer's disease. Zinc complexes, especially



**Scheme 1.3** Stereochemical possibilities observed with polylactide synthesis.

zinc(II)lactate, are a plausible replacement with low toxicity and activities of the same order as aluminum complexes [18]. Zinc powder may also be used but then the active species has been identified as zinc(II)lactate in the preparations of polylactide [19]. Iron salts and particularly iron(II)lactate show comparable activity, but prolonged reaction times mean that some racemization occurs in the synthesis of high molecular weight (50,000 Da) poly(L-lactide) [20].

As can be seen in Scheme 1.3, polylactides can exist in isotactic, syndiotactic, and heterotactic blocks. The configuration has obvious consequences for the material properties of the final polymer. While the ring-opening polymerization of L,L-dilactide or D,D-dilactide leads to isotactic polymers, the polymers of the *rac*-dilactide should consist mainly of isotactic diads. This is due to the fact that *rac*-dilactide is commonly used as a mixture of D,D- and L,L-dilactide with very little *meso*-dilactide content. The formation of syndiotactic diads is expected in the case of *meso*-dilactide polymerization, but the longer range sequence structure of such polymers is typically atactic. Due to the expense of producing stereopure dilactides, a kinetic resolution procedure was developed whereby chiral SALEN (salicylimine) ligands in combination with aluminum isopropoxide catalyst produced isotactic polylactides from *rac*-dilactide. Optical purities were high at 50% conversion; kinetics show that the catalyst system has a 28:1 preference toward one isomer. By choosing the appropriate SALEN ligand enantiomer, selective polymerization of either L,L- or D,D-dilactide could be achieved [21]. Similar approaches using SALEN ligands have been employed to produce syndiotactic and heterotactic polylactides [22].

Tin(II)octoate is also the most common catalyst used for the polymerization of cyclic lactone monomers such as ε-caprolactone; although the mechanism of polymerization may differ [23]. In addition, rare-earth metal complexes have been shown to work as effective catalysts leading to high molecular weight polylactones with low polydispersity [24, 25]. An efficient cationic ring-opening polymerization of lactones has been developed using scandium trifluoromethanesulfonate as catalyst. Poly(ε-caprolactone) with narrow polydispersity and a molecular weight in the order of $10^4$ Da was produced in quantitative yield after 33 h at room temperature in toluene. Only 0.16 mol% of catalyst was required. Similar results were obtained for poly(δ-valerolactone). Notably, the reaction was relatively tolerant to the presence of moisture and other contaminants [26].

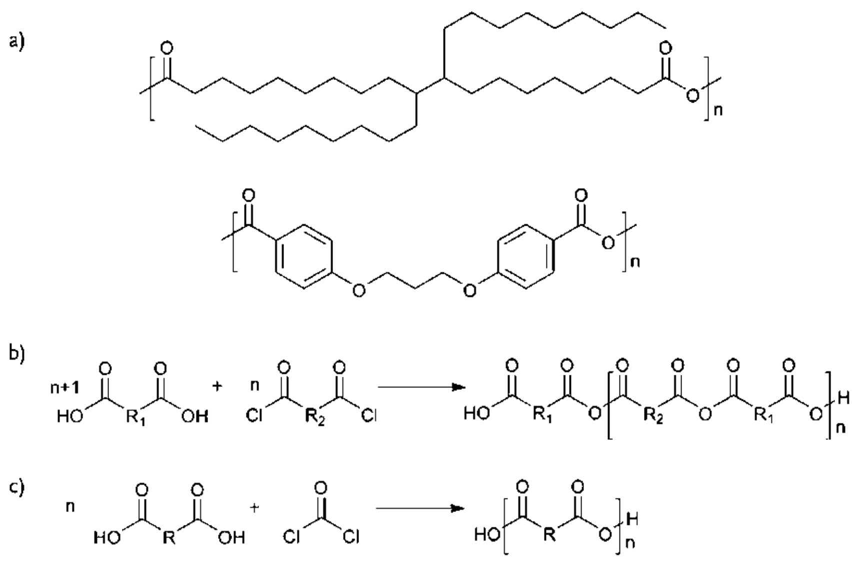
### 1.2.2
### Metal-Free Synthetic Processes

The use of low molecular weight organic molecules to catalyze ring-opening polymerization is a rapidly expanding field [27]. Organocatalytic routes toward polyesters typically involve a nucleophilic polymerization mechanism; problems with side reactions are minimal and products with high molecular weights and very narrow polydispersities can be formed. In addition, organocatalysts may be significantly more abundant and less toxic than metal containing catalysts. These factors point toward future large-scale synthesis applications. Many examples are based around traditional acyl substitution catalysts such as phosphines [28], and pyridine-derivatived nucleophiles [29]. In more recent developments, *N*-heterocyclic carbenes have shown great promise, allowing the synthesis of polylactides with molecular weights up to $10^4$ Da [30]. Supramolecular catalysts are known, which can stabilize transition-states in a noncovalent fashion and thus can exert a great effect on reaction rate and mechanism. To this end, thiourea-containing supramolecular catalysts have shown excellent promise [31].

The enzyme-catalyzed synthesis of polyesters is another technique that is being developed as a very ecologically friendly process with several benefits over conventional chemical polymerization [32]. Enzymatic reactions are often extremely regio- and stereospecific, so unwanted side reactions can be largely eliminated. Lipase-catalyzed ring-opening polymerization has been applied to many substrates including a wide range of lactones and lactides. As an example, poly(D,L-lactide) with molecular weights up to $10^5$ Da could be synthesized in bulk, but recovery yields were relatively low [33]. Among the problems associated with enzymatic polymerization are the high cost of enzymes, long reaction times, and relatively low molecular weight products. These challenges are to be met before enzymes can be used for industrial scale synthesis.

### 1.2.3
### Polyanhydrides

Polyanhydrides are an important class of biodegradable polymers which are closely related to the polyesters [34, 35]. Monomers used are commonly hydrophobic long chain fatty-acid-derived diacids or aromatic group containing diacids such as shown in Scheme 1.4a. Polyanhydrides are treated in detail in Chapter 3. They are



**Scheme 1.4** Typical polyanhydrides and synthetic methods.

mentioned here for comparative reasons, as they typically degrade in an alternative manner to poly(hydroxycarboxlic acids) due to their hydrophobic nature (Section 1.4).

Aliphatic diacids can be polycondensated to polyanhydrides by reaction with acetic acid anhydride. The reaction proceeds in two steps. First of all, oligomeric polyanhydrides with terminal acetate groups are received, further reacting to high molecular weight products at elevated temperatures and under vacuum. Using catalysts like cadmium acetate in the second step, average molecular weights of $10^5$ Da are reached. Under comparable conditions, glutaric acid and succinic acid form cyclic monomers in contrast to sebacic acid. The reaction of dicarboxylic acids and diacidchlorides results in poor molecular weight products (Scheme 1.4b). A method to gain high molecular weight products even at low temperatures is the use of phosgene as condensation agent (Scheme 1.4c). Formed hydrochloric acid in the reaction is sequestered and removed from the growing polymer by use of insoluble proton scavengers.

## 1.3
## Physical Properties

### 1.3.1
### Crystallinity and Thermal Transition Temperatures

As shown in Table 1.1, high molecular weight polyglycolides, polylactides, and copolymers thereof are typically strong, stiff materials with high modulus (*E*) and

**Table 1.1** Material properties of various poly(lactide-*co*-glycolides) [5].

| **Comonomer proportion (mol%)** | | | **Polymer properties** | | | | |
|---|---|---|---|---|---|---|---|
| **Diglycolide** | **L,L-dilactide** | ***rac*-Dilactide** | **$T_g$ (°C)** | **$T_m$ (°C)** | **$E$ (GPa)** | **$\sigma_B$ (MPa)** | **$\varepsilon_B$ (%)** |
| 0 | 100 | 0 | 57 | 174 | 3.6 | 58 | 2.1 |
| 0 | 84 | 16 | 55 | 124 | | | |
| 0 | 75 | 25 | 60 | – | 3.4 | 46 | 1.6 |
| 0 | 50 | 50 | 60 | – | 3.3 | 46 | 3.2 |
| 0 | 25 | 75 | 59 | – | 2.8 | 41 | 2.9 |
| 0 | 0 | 100 | 59 | – | 3.2 | 48 | 8.7 |
| 100 | 0 | 0 | 36 | 228 | 7.0 | | 15.0 |
| 90 | 10 | 0 | 37 | 200 | | | |
| 75 | 25 | 0 | 44 | – | | | |
| 50 | 50 | 0 | 44 | – | | | |
| 25 | 75 | 0 | 52 | – | | | |
| 75 | 0 | 25 | 43 | – | | | |
| 25 | 0 | 75 | 54 | – | | | |

tensile strength ($\sigma_B$). These properties are of similar magnitude to those found within human hard tissues (bones, ligaments, tendons) [36], and are useful for the biomaterial applications mentioned in Section 1.1.1.

Polyglycolide is of high crystallinity, 40–55%, and has a relatively high melting point of 228 °C. The glass transition temperature is 36 °C. Polyglycolide is insoluble in most organic solvents with the exception of highly fluorinated solvents, which must be taken into account when processing materials. Upon copolymerization with dilactides, amorphous materials are produced if the diglycolide content is less than 25%. The glass transition temperature rises from 36 °C to 54 °C as the amount of dilactide monomers are incorporated into the polymer. Poly(L-lactide) has slightly lower crystallinity of 37%; $T_g = 57\,°C$ and $T_m = 174\,°C$. Incorporation of *rac*-dilactide as a comonomer gradually decreases the crystallinity and at 25% *rac*-dilactide content amorphous polymers result. Poly(D,L-lactide) with a glass transition temperature of 65 °C is completely amorphous. The bacterially produced poly(3-hydroxybutyrate) is highly crystalline at 60–80% and has $T_g = 10\,°C$ and $T_m$ of 179 °C. The modulus is 3.5 GPa. As shown in Table 1.2, incorporation of 3-hydroxyvaleric acid as comonomer leads to a softer and more elastomeric material [37].

Materials comprised of the other major groups of poly(hydroxycarboxylic acid) are considerably softer and more elastic. The polyetherester polydioxanone has a melting point of 115 °C and a glass transition temperature in the range of −10–0 °C. The crystallinity is approximately 55%. Polydioxanone has a lower modulus (1.5 GPa) than the polylactide materials, and loses mechanical strength at a higher rate during hydrolytic degradation. Poly(ε-caprolactone) is a semicrystalline

**Table 1.2** Physical properties of various poly([3-hydroxybutyrate]-*co*-[3-hydroxyvalerate])s [5].

| Amount of 3-hydroxyvaleric acid (mol%) | $T_g$ (°C) | $T_m$ (°C) | $E$ (GPa) | $\varepsilon_B$ (%) |
|---|---|---|---|---|
| 0 | 10 | 179 | 3.5 | 6 |
| 9 | 6 | 162 | 1.9 | |
| 20 | –1 | 145 | 1.2 | |
| 25 | –6 | 137 | 0.7 | |

polymer with a melting point in the range of 59–64 °C. The glass transition temperature is –60 °C. Poly(ε-caprolactone) has a relatively very low modulus (0.4 GPa) but an extremely high elongation at breakage of over 700%. Poly(trimethylene carbonate) is an elastomeric polyester with high flexibility but limited mechanical strength, and is the most commonly employed in copolymers to increase elasticity [38].

### 1.3.2
### Improving Elasticity by Preparing Multiblock Copolymers

While the degradation rate and the degradation behavior of the polymers described previously are adjustable, the mechanical properties of these materials are only of restricted variability. The homopolymers of the α-hydroxycarboxylic acids are highly crystalline, brittle materials. The elongations at break are relatively low compared to polymers such as polyethylene terephthalate ($\varepsilon_R = 100\%$) and polypropylene ($\varepsilon_R = 400\%$) [37]. The mechanical properties are sufficient for the production of fibers [39]. However, in addition to the described polymer systems, elastic, tough materials are desirable. A concept to realize this requirement is the preparation of phase-segregated block copolymers. One segment should be crystallizable and act as crosslinking unit to give the material the desired strength. The second segment should be amorphous, with a low glass transition temperature that is responsible for the elasticity. This principle is shown in Figure 1.1.

One method to generate high molecular weight multiblock copolymers is to the co-condensation of two bifunctional linear prepolymers, known as telechelic polymers [40]. A group of copolyesterurethanes can be used as a case in point [41]. Poly([3-*R*-hydroxybutyrate]-*co*-[3-*R*-hydroxyvalerate])-diol is used as crystallizable segment. It is prepared by transesterification of high molecular weight bacterially produced polyester with a low molecular weight diol. The number average molecular weight, $M_{n,}$ of this telechelic polymer ranges from 2100 to 2500 g mol$^{-1}$. The soft segments are telechelic copolyester diols ($M_n$ = 500–3000 g mol$^{-1}$), prepared by ring-opening polymerization of lactones with a low molecular weight diol. A low molecular weight diisoyanate was used to link the polymer blocks through urethane linkages. In this way, the final polymers may be considered as poly(ester urethane)s. With the correct conditions, products with an average molecular

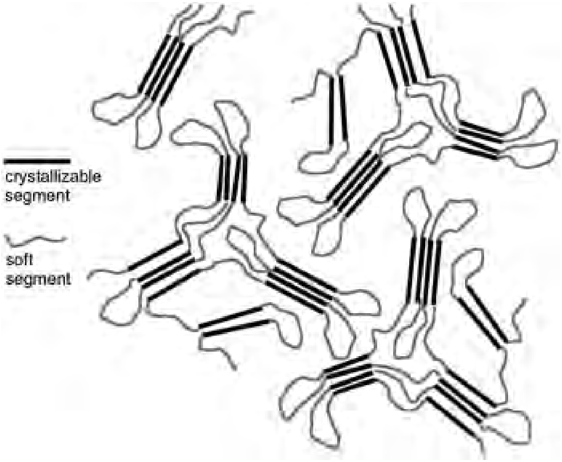


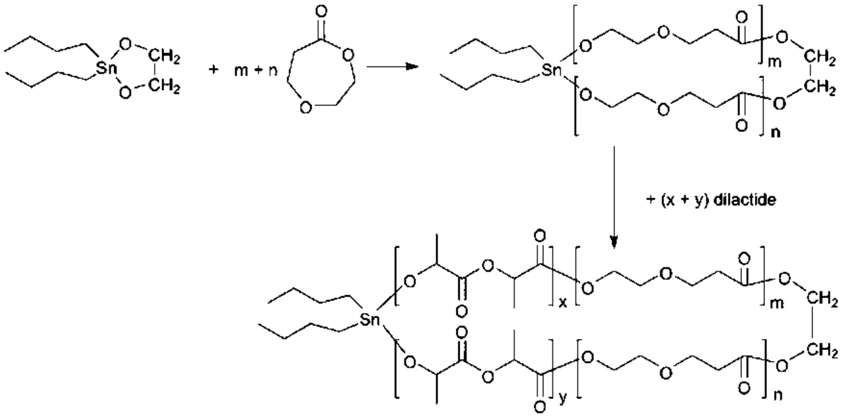
**Figure 1.1** Schematic diagram of the morphology of multiblock copolymers.

**Table 1.3** Influence of the weight content of hard segments in multiblock copolymers on material properties [41].

| Hard segment content (wt%) | $E$ (MPa) | $\varepsilon_R$ (%) | $T_g$ (°C) | $T_m$ (°C) |
|---|---|---|---|---|
| 31 | 60 | 1250 | −24 | 132 |
| 46 | 186 | 1130 | −18 | 134 |
| 61 | 400 | 270 | −10 | 136 |

weight of more than $10^5$ Da were obtained. As can be seen in Table 1.3, lowering the weight percentage of hard segments lowers the material modulus and increases the elongation at break. These polymers have low glass transition temperatures, which prevents them from forming brittle materials at body temperature.

Routes to prepare polyester-based block copolymers have been widely studied [42]. The most direct route is via sequential addition of monomers to systems polymerizing under living conditions. However, this is not broadly applicable to polyester synthesis as the monomers must have comparable reactivities under one set of conditions. This approach is more effective for the copolymerization of similarly functionalized lactones, although the resultant blocks generally have similar physical properties, so phase segregation is not realized [43]. The large difference in reactivity ratio between dilactides and lactones makes it difficult to synthesize such block copolymers. However, an elegant approach using a cyclic tin oxide catalyst has been developed to produce ABA-type triblocks [44, 45]. The ring-expansion mechanism is outlined in Scheme 1.5. This process forms telechelic polymers that can be crosslinked directly with diisocyanates or activated diesters.



**Scheme 1.5** Ring-expansion mechanism for the preparation of ABA triblock polyesters.

### 1.3.3
### Covalently Crosslinked Polyesters

A further method to produce elastomeric polyester-based materials is the preparation of crosslinked amorphous polyesters. In this case, crystalline regions are absent but mechanical strength is given by the inherent rigidity of the network. Such materials are often described as “cured” polymers [46]. Due to the absence of crystalline regions, erosion occurs more homogeneously, and properties can be tailored by composition. Such elastomers were prepared by the photopolymerization of methacrylate functionalized star-shaped poly([ε-caprolactone]-*co*-[*rac*-lactide]) (see Section 1.5.2). Networks suitable for implant materials were obtained, with physical properties adjustable by selecting the molecular weight of the precured polymers [47]. Poly(diol citrates) were synthesized by reacting citric acid with various diols to form a covalent crosslinked network via polycondensation [48]. The physical properties and degradation characteristics could be controlled by choosing different diols and by controlling the crosslink density of the polyester network. Biocompatible materials with elongations at break as high as 500% could be obtained. Other common crosslinkers used for curing polymers are multifunctional isocyanates and acid chlorides.

### 1.3.4
### Networks with Shape-Memory Capability

Polymer networks can be designed in a way that they become capable of a shape-memory effect [49]. Such materials possess the ability to memorize a permanent shape, which can substantially differ from their temporary shape. The transition from the temporary to the permanent shape could be initiated by an external stimulus such as a temperature increase above a characteristic switching temperature of the polymer (thermally induced shape-memory effect). Exemplary shape-memory biodegradable polyesters have been prepared as networks of star-shaped polymers crosslinked by diisocyanates [50], or as photocrosslinkable macrodimethacrylates [51]. Biodegradable shape-memory polymers will be covered in more detail in Chapter 8.

## 1.4
## Degradation Mechanisms

In general, two different mechanisms for the biodegradation of polyesters are discussed in literature: bulk degradation and surface erosion [52]. In the bulk degradation process, water diffuses into the polymer matrix faster than the polymer is degraded. The hydrolyzable bonds in the whole polymer matrix are cleaved homogeneously. Therefore, the average molecular weight of the polymer decreases homogeneously. In the case of surface erosion, the diffusion rate of water into the polymer matrix is slower than the degradation rate of the macromolecules. The degradation only takes place in the thin surface layer while the molecular weight of the polymer in the bulk remains unchanged. Surface erosion is a heterogeneous process, with a rate strongly dependent on the shape of the test sample (e.g., size of the surface) [53].

The majority of polyester materials undergo bulk hydrolysis, as will be explained in the following section. Polyanhydride materials differ from the common polyesters by the fact that they undergo linear mass loss by surface erosion mechanisms [54]. The hydrophobic chains preclude water penetration into the bulk of the material, thus negating bulk erosion mechanisms.

### 1.4.1
### Determining Erosion Kinetics

Erosion rates can be determined *in vitro* and *in vivo* [55]. For *in vitro* experiments, the polymers are exposed to an aqueous solution, in which ionic strength, pH-value, and temperature can be varied. The degradation products of the polymer can be isolated from the aqueous solution and characterized. The addition of enzymes is also possible. Furthermore, the polymers can be exposed to cell- and tissue cultures. By suitable selection and systematic variation of the *in vitro* test conditions, the influence of single parameters on the degradation behavior of the polymer can be determined. Accelerated degradation tests at elevated temperature (usually 70 °C) serve as preliminary experiments for planning the 37 °C experiments and to give reference to the extended degradation behavior of the materials. Another method to accelerate hydrolysis tests is the elevation of the pH-value of the degradation medium to the alkaline region (as a rule 0.01 or 0.1 M NaOH solution). The reaction of cell- and tissue cultures in contact with the material gives information on the compatibility of the partially degraded polymer samples and their degradation products.

*In vivo* experiments are performed with different species such as dogs, monkeys, rats, mice, and sheep [56]. To investigate the degradation behavior, the implants are typically placed subcutaneously or intramuscularly. The tissue compatibility of the polymer can be determined by histological investigations. There are several characteristics to follow in implant material degradation, for example, height, weight, and mechanical properties of the test device. An additional method for *in vivo* tests is marking of the implant with $^{14}C$ or by fluorescent chromophores. In this case, it can be observed where the fragments of the degraded polymer and the degradation products in the test animal remain. Furthermore, the change in thermal properties, crystallinity, and the surface properties (wettability, roughness), depending on the degradation- and implantation time duration, can be determined.

### 1.4.2
### Factors Affecting Erosion Kinetics

Poly(hydroxycarboxylic acid)s degrade via the bulk process [57]. The degradation process can be divided into three parts. In the first step, water is absorbed and the polymer swells. Several ester bonds are cleaved already, but there is no mass loss. In the second step, the average weight is significantly reduced. As ester bonds are cleaved, carboxylic groups are formed, which autocatalyze the hydrolysis. During this period, the polymer loses mechanical strength. The third step is characterized by mass loss of the test sample and an increase in degradation rate. The degradation of an implanted material is completed when oligomeric and low-molecular-weight fragments are dissolved in the surrounding medium. The dissolved polymer fragments are then hydrolyzed to the free hydroxycarboxylic acids. Degradation products, many of which occur naturally within the metabolic cycle (e.g., lactic acid), are typically removed from the body without toxic effect. To some extents, smaller crystalline segments may remain, which are eliminated from the body by phagocytosis [58].

The degradation times of several poly(hydroxycarboxylic acids) are summarized in Table 1.4. The differences in degradation rates may be rationalized mainly by the ability of water to permeate the polymers (crystallinity and hydrophobicity), and in the case of polylactides the presence of an α-methyl group which hinders hydrolysis on steric grounds. Copolymers, due to the greater prevalence of amorphous regions, are generally degraded faster than homopolymers [59].

The higher degradation rate of poly(*rac*-lactide) compared to poly(L-lactide) is due to the higher crystallinity of the isotactic poly(L-lactide). Polyglycolide, being less hindered at the scission site, is degraded relatively quickly. The degradation rate of poly(lactide-*co*-glycolide) can be finely tuned by varying the monomer content. Polydioxanone and poly(ε-caprolactone) are also less sterically hindered but increased hydrophobicity hinders erosion. Poly(ε-caprolactone), with a pentylene ($–C_5H_{10}–$) chain, erodes an order of magnitude slower than polyglycolide.

Due to the high crystallinity, poly(3-*R*-hydroxybutyrate) is relatively slowly degraded. In this case, a certain amount of surface erosion takes place first. With

**Table 1.4** Comparative degradation times of different polyesters in physiological conditions [5].

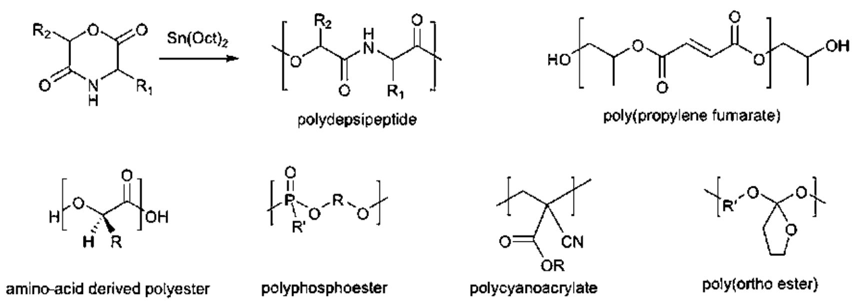
| Polymer | Degradation time (months) |
|---|---|
| Poly(L-lactide) | 18–24 |
| Poly(*rac*-lactide) | 12–16 |
| Polyglycolide | 2–4 |
| Poly(3-hydroxybutyrate) | $\gg$36 |
| Polydioxanone | 6–12 |
| Poly(ε-caprolactone) | >24 |
| Poly([L-lactide]-*co*-glycolide) 50:50 | 2 |
| Poly([*rac*-lactide]-*co*-glycolide) 85:15 | 5 |
| Poly([*rac*-lactide]-*co*-[ε-caprolactone]) 90:10 | 2 |

proceeding degradation, a loss of weight and increasing porosity lead to a bulk degradation. Poly([3-*R*-hydroxybutyrate]-*co*-[3-*R*-hydroxyvalerate]) is hydrolyzed more rapidly due to lowered crystallinity. The degradation of the poly(3-*R*-hydroxybutyrate) can be accelerated by microorganisms and enzymes [60]. Bacterially produced poly(3-*R*-hydroxybutyrate) is degraded faster than synthetic poly(3-*R,S*-hydroxybutyrate), while poly(3-*S*-hydroxybutyrate) is not hydrolyzed at all [61]. Enzymatic digestion proceeds at the surface of the sample as proved by scanning electron microscopy. The enzyme, because of high molecular weight, is too large to diffuse into the bulk of the material. Another important factor to take into account when considering the processing of these polymers is that above 200 °C thermal degradation can occur [62].

## 1.5
## Beyond Classical Poly(Hydroxycarboxylic Acids)

### 1.5.1
### Alternate Systems

In addition to the broadly studied groups described previously, there are some notable examples to include when discussing biodegradable polyesters. Scheme 1.6 shows a selection of such alternate polyester structures. Various amino acids were converted to the corresponding α-hydroxycarboxylic acid and then polymerized to give stereogenic α-substituted polyglycolide analogs [63, 64]. This approach could give access to polymers with more tailored properties and sophisticated material properties. Polydepsipeptides are an interesting class of poly(ester amide) which are copolymers of α-hydroxycarboxylic acids and α-amino acids [65]. They are synthesized from morpholine diones (analogs of diglycolide, in which one lactone is replaced by a lactam group) by many of the same procedures outlined for polyglycolide synthesis. Polydepsipeptides degrade through the ester bonds, whereas the amide linkages remain intact under physiological conditions [66].



Scheme 1.6 Alternative polyesters and closely related polymers.

There is the possibility to select the amino acid component to incorporate functional groups into the chain in a facile manner [67].

Poly(propylene fumarates) are a bulk eroding class of polyester which are synthesized typically via transesterification [68, 69]. Although molecular weights are generally low, the unsaturated polymer backbone can be photochemically crosslinked to provide polymer networks with desired properties for implant materials. The general concept of crosslinking by photopolymerization has been extended to the other polyester classes, typically by methacrylate functional groups, which have been appended to polymeric precursors [70]. Supramolecular polyesters have been developed by attaching self-recognizing binding units on either end of telechelic polylactones [71]. These polymers are shown to self-assemble via noncovalent means into long strands composed of multiple individual blocks. Such systems show a high level of sensitivity to environmental conditions, and as such may be considered as “smart” polyester systems.

Although not strictly polyesters, some interesting analogs remain. Poly(ortho esters) are materials which are studied mainly as drug delivery vehicles [72]. Like polyanhydrides, they have very labile bonds but are hydrophobic in nature. Water is precluded from the bulk polymer, hence retarding degradation. As such, polyorthoesters have fairly linear surface erosion behavior, ideal for controlled drug release. Polyphosphoesters are hydrolytically degraded polymers that have an extra degree of versatility due to the pentavalent nature of the phosphorous atom [73]. They are highly hydrophilic and show good biocompatibility. Copolymers with other esters such as lactides have been prepared and studied for the range of applications from tissue engineering to drug delivery. Poly(alkyl cyanoacrylates) are rapidly degrading polymers which have widespread medical applications [74]. Although they only contain ester units in the side chains, they are degraded not only at the carbonyl position but at the carbon–carbon sigma bond of the polymer backbone chain. This behavior separates this class of material markedly from the polyesters.

### 1.5.2
### Complex Architectures

While the properties of linear polyesters have been widely studied, branched polyesters are becoming the subject of increasing study. The search for more complex architectures is stimulated mainly by the desire to lower crystallinity, increase the amount of endgroups for further functionalization, or to further control degradation behavior.

Star-shaped poly(caprolactones) with molecular weights in the $10^4$ Da range were prepared starting with triol or tetraol multifunctional initiators [75]. These multivalent products were further reacted with glycolide derivatives to give a star-shaped block copolymer of poly([ε-caprolactone]-block-[lactide-*alt*-glycolide]). This globular architecture has a soft segment on the interior with a more crystalline outer sphere. Hyperbranched polyesters of $M_n \approx 10^4$ Da were prepared by copolymerizing polylactide with an alcohol-containing lactone as a latent $AB_2$ monomer [76]. This dramatically changed the material properties of the polymer with reduced crystallinity and lowered glass transition temperature due to the branching. Poly(ether ester) dendrimers were synthesized from lactic acid and glycerol [77]. These structures have perfect branching radiating from a single point, and make an interesting class of polyvalent biocompatible material.

Polysaccharides have been used as a multivalent scaffold, from which to graft polyesters. Polylactide grafted onto pullulan showed a relatively increased degradation rate which was attributed to the branching effect and the increased hydrophilicity of the pullulan core [78]. Comb polymers were prepared by grafting multiple polylactides to a linear polymethacrylate chain [79]. Highly crystalline regions were observed at the interdigitating comb regions, leading to novel material properties. Interesting and complex structures were formed from polylactides attached to ligands which are able to aggregate into defined supramolecular complexes with Cu(I) ions [80]. Size defined nanoparticles resulted.

### 1.5.3
### Nanofabrication

The previous analyses of polyester materials were given largely in the context of mechanical properties required for tissue engineering or medical implant applications. Polyesters with nanoscale dimensions, however, are widely studied as nanovehicular delivery agents for drugs and biodiagnostic molecules [81, 82]. For the "top-down" approach, submicron polyester spheres can be prepared by a range of molding and lithography techniques [83]. In addition, electrospinning techniques are emerging as a powerful tool for the preparation of nanoscale fibers [84]. Biodegradable polyesters may be formulated with inorganic nanomaterials to provide nanobiocomposites which have relatively controllable physical properties [85]. Polyester/clay biocomposites have been the subject of considerable study.

"Bottom-up" approaches allow the preparation of nanoscale materials without the need for further processing. Various nanospheres of polylactides and polylactones were prepared in the size range of 80–200 nm using a miniemulsion technique. Endocytic, cellular uptake of fluorescently labeled particles was observed, with kinetics revealing that polyesters are endocytozed much faster than polystyrene particles [86]. Polylactide nanoparticles presenting mannose residues at their surface were prepared with dimensions of 200–300 nm by a nanoprecipitation technique [87]. Biochemical assays were used to quantify their recognition of lectin proteins. Such nanoparticles are designed to be specifically recognized by mannose receptors, which are highly expressed in cells of the immune system. Future applications as vaccine delivery agents are anticipated. Thiol-capped polylactides were used to coat photoluminescent quantum dots, which are designed as drug delivery nanovehicles with both diagnostic imaging properties and controlled drug release properties [88]. Another route to nanoscale particles is via micelle formation of amphiphilic block copolymers [89]. Polyethylene glycol-block-lactides were shown to form polymeric micelles, into which paclitaxel as a model drug was encapsulated. Biodistribution and drug release behaviors were studied.

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# 2

# Biotechnologically Produced Biodegradable Polyesters

*Jaciane Lutz Ienczak and Gláucia Maria Falcão de Aragão*

## 2.1

## Introduction

Polyhydroxyalkanoates (PHAs) are polyesters synthesized by many microorganisms as a carbon and energy storage material [1].

The interest in establishing PHA as an alternative plastic to conventional petrochemical-based plastics was first motivated because it can be produced from renewable carbon sources and since they are biodegradable. Fuel-based polymers are extensively used due to their easy manufacturing and low cost of production. Unfortunately, these same qualities can transform them into an important environmental problem because they are cheap and disposable. The great demand for this kind of polymer production generates pollution and problems related with the disposal in landfills because these materials are resistant to degradation [2]. In response to rising public concern regarding the effects of fuel-based materials in the environment, biopolymers are a reality that can minimize these problems. Biopolymers are polymeric materials structurally classified as polysaccharides [3, 4], polyesters [5–7], or polyamides [8]. The main raw material for manufacturing them is a renewable carbon source, usually carbohydrates such as sugar cane, corn, potato, wheat, beet, or a vegetable oil extracted from soybean, sunflower, palm, or other plants. Currently, biopolymers of interest include thermoplastic starch [9], polylactides (PLA) [10], xanthan [3], polyamides cyanophycin, and the PHA class which includes the most studied biopolymer, poly(3-hydroxybutyric) (P[3HB]) and its copolymer poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (P[3HB-*co*-3HV]) [7, 11, 12].

PHAs are able to replace synthetic polymers because they have very similar properties with the advantage of being completely degradable to water and carbon dioxide in aerobic conditions [13]. Depending on the monomer composition, the properties of PHA polymers can range from thermoplastics to elastomeric. P(3HB) shows thermoplastic and mechanical properties similar to those of polypropylene [13, 14]. Despite the possibility of PHA applications in medicine, pharmaceutical, food, and chemical industries as an alternative to conventional plastic

*Handbook of Biodegradable Polymers: Synthesis, Characterization and Applications,* First Edition. Edited by Andreas Lendlein, Adam Sisson.

[15], biodegradable plastics still have minimal participation in the market because of the high cost compared to fuel-based polymers. Therefore, many research groups are conducting studies to reduce the production costs of biopolyesters by using low-cost substrates [11, 16–19], large-scale fermentation methods [19–22], and metabolic engineering to develop strains with higher productivity and capable of assimilating renewable carbon sources. In addition, the PHA granule has various protein-based functions and has attracted interest due to the utilization of these bionanoparticles in medical and biotechnological applications [23, 24].

## 2.2
## History

Beijerinck [25] observed granules in a microscope inside *Rhizobium* cells. Such granules were present in the "bacteroides" isolated from nodules and were described as being extremely refractile globules. Another microbiologist, Lemoigne [26], noticed that, when cultures of *Bacillus subtilis* were followed by autolysis in distilled water, the pH value decreased because of the formation of an unknown acid. This acid was subsequently identified as monomer of poly-β-hydroxybutyric acid [27]. In the same period, Stapp [28], analyzing the results of other researchers, suggested that *Azotobacter chroococcum* inclusions could be easily extracted with chloroform, and identified this structure as poly-β-hydroxybutyrate. In 1958, the functional P(3HB) pathway was proposed by Macrae and Wilkinson [29]. They observed that *Bacillus megaterium* stored the polymer especially when the ratio glucose/nitrogen in the medium was high, and that the subsequent degradation occurred quickly with the absence of the carbon source. PHA's potential usefulness has been recognized since the first half of the 1960s through patents related to P(3HB) production process [30]; extraction from the producing biomass [31]; plasticization with additives [32]; the use unextracted as a polymer mixed with other cell material [33]; and pure for absorbable prosthetic devices [34]. In a review about the regulatory role and energy resource microorganisms, published in 1973 by Dawes and Senior [35], P(3HB) was found to be a microbial resource material storage as starch and glycogen. In the period between 1974 [36] and 1989 [37], other hydroxyalkanoates (HAs) have been identified besides 3HB, such as 4-hydroxybutyrate (4HB), 3-hydroxyhexanoate ($3HH_x$), 3-hydroxyoctanoate (3HO), 3-hydroxyvalerate (3HV), among others. The identification of copolymer poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (P[3HB-*co*-3HV]) has led to a positive impact on research and commercial interest because the homopolymer (P[3HB]) is brittle and has a low extension break. This lack of flexibility limits its range of application in relation to the copolymer, which has a much lower melting point and is less crystalline [38]. The industrial production of these polymers began in 1980 by the UK chemical group Imperial Chemical Industry (ICI) [39]; after that, it started to be produced by others industries (Table 2.1).

In the past few decades, PHA researchers have been living a period of interest for metabolic engineering [40], and site-directed mutagenesis of the enzymes

**Table 2.1** Summary of industrial PHA production: past to present.

| Industry | History | Product |
|---|---|---|
| Chimie Linz | Currently Biomer | P(3HB) |
| Biomer | Production from sucrose | P(3HB) |
| Mitsubishi | Production from methanol, with the name Biogreen. | P(3HB) |
| PHB Industrial | Production from sugar cane sucrose, with the name Biocycle. | P(3HB-*co*-3HV) |
| Zeneca Bioproducts (previously ICI). | A development program has been carried out (1980–1990) for PHA commercialization (Biopol). Biopol is a copolymer family P(3HB-*co*-3HV) produced by microorganisms using glucose and propionic acid as substrates. Zeneca has begun P(3HB) and P(3HB-*co*-3HV transgenic plant production. | P(3HB-*co*-3HV) |
| Monsanto | Zeneca was incorporated by Monsanto in 1996 and has continued the development of this polymer. However, in 2001 Monsanto stopped their work. | P(3HB-*co*-3HV) |
| Metabolix | *Spin off* Industry. Nowadays possesses an agreement with Archer Daniels Midland Co (ADM) for copolymer production in industrial scale. | P(3HB-*co*-3HV) |
| Procter & Gamble | Nodax is a copolymer family produced from glucose and vegetable oils, above all palm oil. | P(3HB-*co*-3HHx) |
| Kaneka Co | Licensed by Procter & Gamble for Nodax production in industrial scale. | P(3HB-*co*-3HHx) |

Adapted from "Biopolímeros e Intermediários Químicos" [40].

involved in PHA biosynthesis will most likely result in new polyesters [41]. In the 1980s, the first study for PHA production from recombinant microorganisms involving cloning of PHA biosynthetic genes [42, 43] was realized, and in the 1990s it was also possible to study transgenic plants as potential producers of PHA in the future [44–47]. Many studies have been made to determine tertiary and quaternary structures of PHA synthesis, which would allow researchers to understand the catalytic mechanisms, the substrate specificities of this group of enzymes, and probably also the factors that determine the molecular weight of produced PHA [11, 48–50]. Interest in granule-associated protein, large-scale production, and high productivity has also been noted, besides new methods of PHA recovery. In this history, it can be observed a scientific PHA evolution in eight decades of research and process development for biodegradable polymer production, initially recognized by lipophilic inclusions and currently being studied at molecular level by protein and metabolic engineering.

## 2.3
## Polyhydroxyalkanoates – Granules Morphology

PHA occurs as an insoluble inclusion in the cytoplasm. Several structural models of granules have been proposed with the purpose of answering the questions related to granule formation, structure, size, and composition. In this context, the first structure, shown by Ellar [51], was a fibrilar PHA structure of granules with 10–15 nm in length and enclosed by a membrane approximately 2–4 nm thick. The same author proposed a granule composition of approximately 98% PHA and 2% proteins. In another study, Lundgren [52] suggested that the composition of the granule has a membrane with approximately 0.5% and 2% of lipid and protein, respectively, and not only proteins.

Dunlop and Robards [53] investigated the structure of P(3HB) granules in *Bacillus cereus* using freeze-etching methods and found that the granule has a central core amounting to 50% of granule volume and an outer coat with different densities. Ballard and coworkers [54] reported many responses in relation to the size and number of the granules of P(3HB) in *Alcaligenes eutrophus* (nowadays, *Cupriavidus necator*) by freeze-fracture and by using a cylindrical cell model to interpret the results. These authors proposed a granule diameter from 0.24 to 0.5 μm and the average number of granules per cell remained constant at 12.7 ± 1.0 and 8.6 ± 0.6 for two different scale experiments. Another important consideration by these authors was that the number of granules is fixed at the earliest stages of polymer accumulation and polymer accumulation ceases when a P(3HB) content of about 80% is attained, although PHA synthase activity remains high.

Initial studies about PHA characteristics inside the granule were developed in the 1960s through X-ray studies of solid P(3HB) and the results led initially to the conclusion that P(3HB) granules *in vivo* were crystalline [55, 56]. Nevertheless, Kawaguchi and Doi [57] have examined by X-ray diffraction the structure of native P(3HB) granules of *A. eutrophus* and concluded that the polymer presented an amorphous state. These authors concluded that the treatment of granules with alkaline hypochlorite, sodium hydroxide, aqueous acetone, or lipase initiated crystallization of P(3HB) by removing lipid components. Hence, the initial studies of the PHA granule structure proposed a noncrystalline structure, with the presence of fluid polyesters and a small amount of phospholipids and proteins, but there was no knowledge about the protein type and granule formation.

In a previous study about depolymerase, Foster *et al.* [58] observed serine residues in the active site of a functional depolymerase (PhaZ, structural gene *pha*Z) associated with isolated poly(3-hydroxyoctanoate) (P[3HO]) granules, which is a copolymer obtained by feeding *Pseudomonas oleovorans* with *n*-octanoic acid. Following this investigation, Foster *et al.* [59] studied with more details the presence of depolymerase in P(3HO) granules. The results revealed that (i) the *P. oleovorans* depolymerase remains active in isolated P(3HO) inclusion bodies; (ii) this enzymatic activity occurs in association with the organized protein lattice that encompasses the stored P(3HO) polymer; and (iii) depolymerase activity of isolated native P(3HO) granules showed a maximum degradation rate of 1.17 $\mathrm{mg\,h^{-1}}$ at an optimum pH of 9.

Phasins (PhaP, structural gene *pha*P) are defined as a protein class that has a similar role as oleosins of triacylglycerol inclusions in seeds and pollen of plants [60]. These proteins have been identified from *A. eutrophus* (nowadays, *C. necator*) [61] and *Rhodococcus ruber* [62], and have been shown to influence the size of intracellular PHA granules. Phasins have been suggested to have a role as amphiphilic proteins (substance readily soluble in polar as well as in nonpolar solvents) in the interphase between the hydrophilic cytoplasm and the hydrophobic PHA molecule, and may also act as an anchor for the binding of other proteins such as PHA synthase [62]. Inside this group, the GA13 protein, studied by Schembri and coworkers [63], can be noted. These authors studied *Acinetobacter* RA3123, RA3849, RA3757, RA3762, and *Escherichia coli* DH5α to identify the 13-kDa PHA (GA13) granule-associated protein as the protein encoded by a structural gene located within the *Acinetobacter pha locus*. When the P(3HB) granule samples were examined, a protein of approximately 13 kDa (GA13) was identified in all four *Acinetobacter* P(3HB)-positive strains, shown to be the product of the $phaP_{\mathrm{AC}}$ (gene encoded PhaP protein by *Acinetobacter*) gene in strain RA3849 and revealing the presence of two regions containing predominantly hydrophobic and amphiphilic amino acids. This may be involved in the anchoring of this protein into the phospholipid monolayer surrounding the PHA granule. *E. coli* showed a small amount of accumulated P(3HB), however, with large-sized granules. This fact may be related to the poor expression of GA13 protein in this strain, which is able to reduce PHA synthase (PhaC, structural gene *pha*C) activity.

Stuart *et al.* [64] reported that different microorganisms (*Ralstonia eutropha*, *Norcadia corallina*, *Azotobacter vinelandii*, and pseudomonads species) showed a different granule protein boundary in electron microscopy and SDS-PAGE. These results can be very interesting for biotechnologists since they indicate a natural "packaging" of polymer during biosynthesis. Maehara *et al.* [65] proposed a structural PHA granule model determining the distinct target DNA sequences for PhaR (a repressor protein which regulates PHA synthesis) binding and demonstrated that PhaR binds not only to DNA but also to PHA. These results confirm that PhaR has bifunctional characteristics, namely, binding abilities toward both PHA and DNA. PhaR is the first protein that interacts directly with PHA polymer. The recognition requirement for this interaction was relatively nonspecific, because PhaR bound to all forms of P(3HB) – crystalline, amorphous, and 3HB oligomers. PhaR recognizes and binds directly to the PHA polymer chains being synthesized, and then the expression of PhaP is initiated at the onset of dissociation of PhaR from an upstream element for *pha*P. During the elongation of PHA polymer chains, the PHA granules enlarge in size, and then the surfaces of PHA granules become covered by PhaP and other specific proteins before the other nonspecific proteins bind to the PHA granules. Under these conditions, the authors concluded that PhaR is a sensor for PHA synthesis in the cell.

According to the conventional classification of PHA granule-associated proteins proposed by Steinbüchel *et al.* [66], the following four distinct proteins can be defined functionally: class I comprises the PHA synthases, which catalyze the polymerization of the monomers of hydroxyacyl-CoA; class II comprises the PHA depolymerases, which are responsible for the intracellular degradation and mobilization of PHA; class III comprises the phasins (designated as PhaP), which probably form a protein layer at the surface of the PHA granule with phospholipids, lipids, and other proteins; and class IV comprises all other proteins. Figure 2.1 shows a likely model for the PHA granules.

Based on these observations, two models have been proposed for granule formation. The first one is the micelle model, in which the extended PHA chains covalently attached to the synthase aggregate initially into a micelle structure [56, 67]. The physical properties of the polymer are thus proposed to be the driving force for inclusion formation. The second model is the maturing model that was proposed by Stubbe and Tian [68], in which the hydrophobic synthase binds to the inner face of the plasma membrane, leading to a granule surface covered with a lipid monolayer. In this model, the biology of the system and the physical properties of the polymer are required for granule formation.

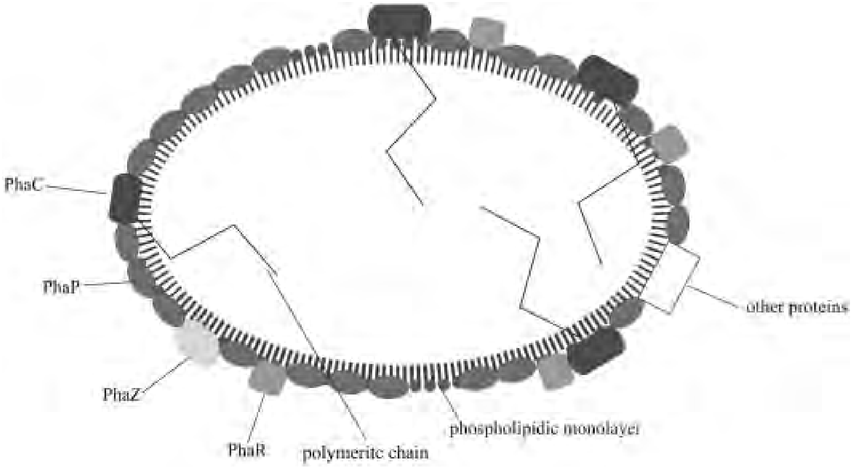

**Figure 2.1** Model for the PHA granules. PhaC protein is a PHA synthase, PhaP protein is a phasin, PhaZ protein is a PHA depolymerase, PhaR protein is a sensor for PHA synthesis in the cell, the phospholipidic monolayer, and other proteins that surround the granule (based on [65], with modifications).

## 2.4
## Biosynthesis and Biodegradability of Poly(3-Hydroxybutyrate) and Other Polyhydroxyalkanoates

### 2.4.1
### Polyhydroxyalkanoates Biosynthesis on Microorganisms

Various microorganisms can accumulate a large amount of PHA inside their cells in response to the limitation of an essential nutrient. Many previous works were concerned with the control of the synthesis of PHA under unbalanced growth conditions [7].

Since the discovery of PHA-producing microorganisms by Lemoigne in 1925, there are over 300 types of microorganisms that accumulate PHA, belonging to the genus *Alcaligenes*, *Azobacter*, *Pseudomonads*, methylotrophs, and some recombinant microorganisms such as *E. coli* [13]. The Gram-negative bacteria *C. necator* has been the most widely used microorganism for the production of P(3HB). *C. necator* was previously categorized as *Hydrogenomonas eutropha, A. eutrophus, R. eutropha,* and *Wautersia eutropha* [69]. *C. necator* has also been used for the commercial production of P(3HB) by many industries [18, 34, 40].

Among the substrates required for PHA production, the carbon source has a primal significance in the case of P(3HB) production, since P(3HB) is composed only of C, H, and O atoms [70]. Microorganisms have the ability to produce PHA from various carbon sources including inexpensive and complex waste effluents. In the past years, our group has prepared works [11, 71–74] in order to reduce the production costs of P(3HB) and its copolymers by the use of renewable carbon sources.

Figure 2.2 shows P(3HB) (a $\text{PHA}_{\text{SCL}}$ – short chain length PHA) production by *C. necator* in two phases: balanced growth and unbalanced growth (Pathways I and II, respectively); P(3HB-*co*-3HV) production from propionigenic substrates by *C. necator* (Pathways II and III); and $\text{PHA}_{\text{MCL}}$ (medium chain length PHA) production from fatty acid *de novo* biosynthetic route and fatty acids β-oxidation (Pathways IV and V, respectively) according to the substrate.

P(3HB) production by *C. necator* occurs in two phases. The first phase comprises the exponential growth where all nutrients are present (balance growth – Pathway I in Figure 2.2) and the second phase shows a nutritional limitation of N, P, S, Mg, or $O_2$ in the presence of an excessive carbon source (unbalanced growth – Pathway II in Figure 2.2) [75]. Hence, the metabolism for the biomass production during balanced growth catabolizes carbohydrates via the Entner–Doudoroff pathway to pyruvate, which can be converted through dehydrogenation to acetyl-CoA. During reproductive growth (Pathway I), acetyl-CoA enters the tricarboxylic acid (TCA) cycle, releases CoASH, and is terminally oxidized to $CO_2$ generating energy in the form of ATP, reducing equivalents (NADH, NADPH, and $FADH_2$) and biosynthetic precursors (2-oxoglutarate, oxalacetate) [76]. Direct amination or transamination of the oxalocetate leads to the synthesis of amino

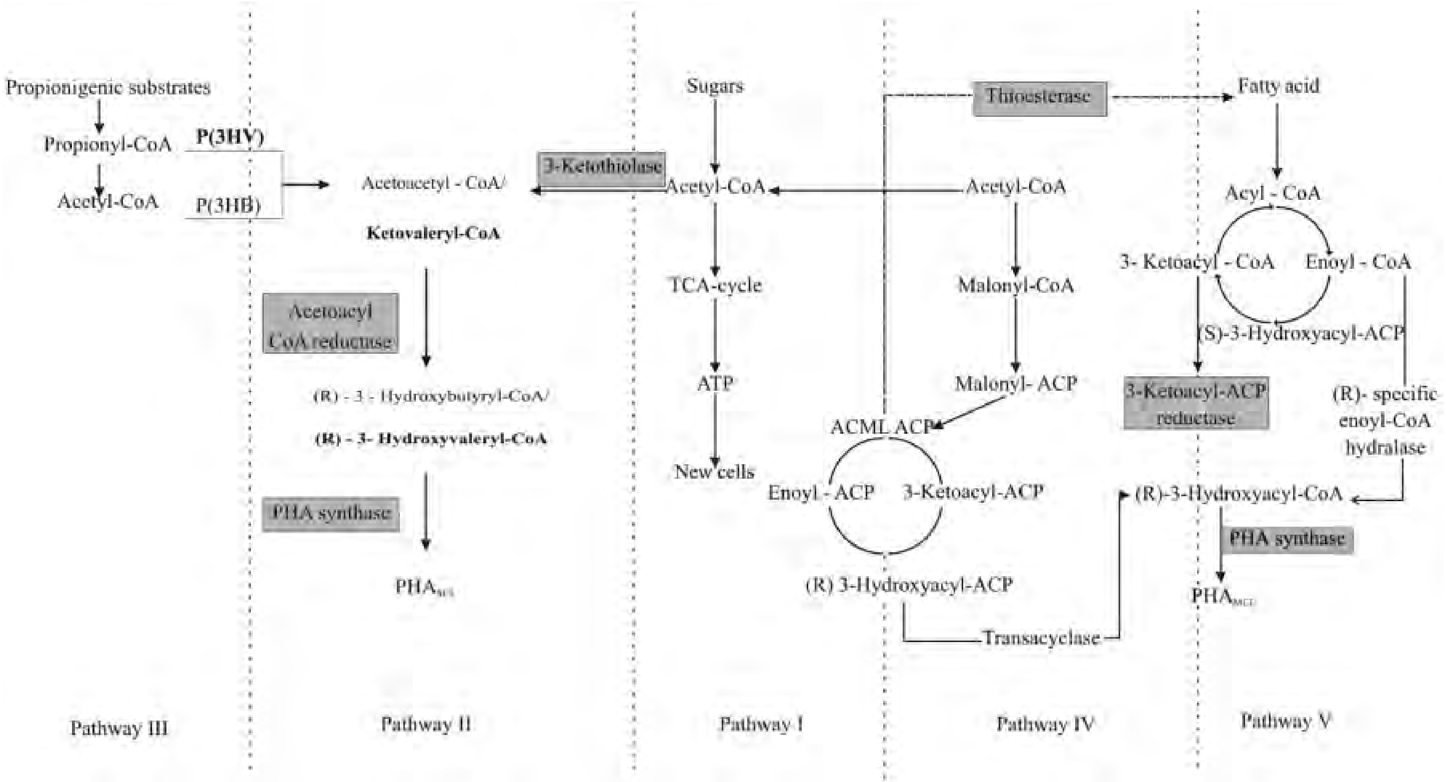

**Figure 2.2** PHA production at pathways proposed for *C. necator*: (I) balanced growth for biomass production; (II) unbalanced growth for P(3HB) (a $PHAS_{SCL}$) production; (III) P(3HB-*co*-3HV) production from propionigenic substrates by *C. necator*, and proposed for pseudomonads; (IV) $PHA_{MCL}$ or $PHAS_{SCL}$ production from fatty acids *de novo* synthesis; and (V) $PHA_{MCL}$ production from fatty acids β-oxidation (based on [83] with modifications).

acids, which are incorporated into the polypeptide chains of nascent proteins. The rate of admission of acetyl-CoA into TCA cycle is dependent on the availability of sources of nitrogen, phosphorous, and other elements, as well as on the oxidative potential of the environment [6].

In Figure 2.2, Pathway II, limitations in nitrogen, phosphorous, oxygen [77, 78], magnesium, or sulfate [79] lead to P(3HB) production. This limitation causes cessation of protein synthesis leading to high concentrations of NADH and NADPH resulting in an inhibition of citrate synthase and isocitrate dehydrogenase and in a slowdown of the TCA cycle and the channeling of acetyl-CoA toward P(3HB) biosynthesis [35]. Acetyl-CoA no longer enters the TCA cycle at the same rate and instead is converted to acetoacetyl-CoA by 3-ketothiolase, the first enzyme of the P(3HB) biosynthetic pathway, which is inhibited by CoA.

According to Figure 2.2, Pathway II, three enzymes are involved in $\text{PHA}_{\text{SCL}}$ production: 3-ketothioloase; acetoacyl-CoA reductase, and PHA synthase. The role of these enzymes is described below.

The first step for PHA formation is catalyzed by *3-ketothiolase*. Its mechanism includes two partial reactions which result in a condensation of two acetyl-CoA molecules to obtain acetoacyl-CoA. Two cystein residues are present in the active site of this enzyme and are responsible for the acetyl-CoA molecule ligament at the enzyme and for the activation of a second molecule of acetyl-CoA, hence entailing a condensation and formation of acetoacyl-CoA [1]. The enzyme catalyzes the reversible reaction shown in Eq. (2.1):

$$2\ \text{Acetyl-CoA} \leftrightarrow \text{acetoacyl-CoA} + \text{CoASH} \tag{2.1}$$

This 3-ketothiolase competes for acetyl-CoA with many other metabolic pathways, including acetate, citrate, and the fatty acids synthesis. This enzyme is inhibited by free CoASH molecules [80].

*Acetoacyl-CoA reductase* catalyzes the second step on PHA biosynthesis (Eq. (2.2)), converting acetoacyl-CoA into hydroxyacyl [1]:

$$\text{Acetoacyl-CoA} + \text{NADPH} + \text{H}^{+} \leftrightarrow \text{3-hydroxyacyl-CoA} + \text{NADP}^{+} \tag{2.2}$$

Two acetoacyl-CoA reductase types, with different specificities for substrates and coenzymes, were found in *C. necator*. The NADH-dependent enzyme is active in D(−) and L(+) substrates, while a NADPH-dependent one is stereospecific or active only at C4 to C6 D(−)3-hydroxyacyl-CoA substrates. During the P(3HB) synthesis, acetoacetyl-CoA is reduced to D(−)3- hydroxybutyryl-CoA, catalyzed by NADPH-dependent enzymes [5].

*PHA synthase* is the key enzyme for PHA biosynthesis. This enzyme catalyzes ester formation through the polymerization of D(−)3-hydroxyacyl-CoAs units, resulting in the polymer. The wide monomer variety that composes PHA is related to large substrate PHA synthase specificities. In this context, *C. necator* PHA synthase is able to polymerize 3-hydroxy, 4-hydroxy, and 5-hydroxyalkanoates from the 4 and 5 carbon hydroxyacyl-CoA, D-isomers [5, 12]. This enzyme is shown in two forms – a soluble form in the cytoplasm (balanced growth) and associated with P(3HB) granules (unbalanced growth) [5].

Based on the types of monomer incorporated into PHA, various metabolic pathways have been shown to be involved in the generation of these monomers [12, 81].

Biosynthesis of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) P(3HB-*co*-3HV) requires, besides 3HB-CoA (3-hydroxybutyryl-CoA), also 3-hydroxyvaleryl-CoA (3HV-CoA). The latter is also required if other copolyesters containing 3HV or poly(3HV) homopolyester are synthesized. 3HV-CoA ([R]-3-hydroxyvaleryl-CoA) is obtained from the condensation of acetyl-CoA and propionyl-CoA into 3-ketovaleryl-CoA (Figure 2.2, Pathways II and III) and a subsequent reduction in the condensation product to 3HV-CoA. The specific substrates for P(3HB-*co*-3HV) production can be propionic acid [7, 11, 82], valeric acid, heptanoic acid, or nonanoic acid. Steinbüchel and Lütkte-Eversloh [12] cited many other sources for poly-(3HV) production. Among them, *n*-pentanol (metabolized from *Paracoccus denitrifricans*), valine, isoleucine, threonine, and methionine are considered precursor substrates for 3HV containing PHA.

Fatty acid *de novo* biosynthesis (Figure 2.2, Pathway IV) is the main route during growth on carbon sources, like gluconate, acetate, or ethanol, that are metabolized to acetyl-CoA, for the $\mathrm{PHA_{MCL}}$ synthesis by pseudomonads like *Pseudomonas putida, P. aeruginosa, P. aureofaciens, P. citronellolis,* and *P. mendocina* [81, 83, 84]. From the results of labeling studies, nuclear magnetic resonance spectroscopy, and gas chromatography mass spectroscopy ([85, 86], cited by [84]), authors concluded that the precursors of $\mathrm{PHA_{MCL}}$ biosynthesis from simple carbon sources are predominantly derived from (*R*)-3-hydroxyacyl-ACP intermediates occurring during the fatty acid *de novo* biosynthetic route. Since the constituents of P(3HB) and PHA represent the *R* configuration and since $\mathrm{PHA_{SCL}}$ and $\mathrm{PHA_{MCL}}$ synthases are highly homologous, the intermediates in fatty acid metabolism are presumably converted to (*R*)-3-hydroxyacyl-CoA before polymerization (Figure 2.2, Pathway V). Nevertheless, some other routes of PHA synthesis are also possible. Other conceivable alternatives are the release of free fatty acids by the activity of a thioesterase with a thiokinase, subsequently activating these fatty acids to the corresponding hydroxyacyl-CoA thioesters or chain elongation with 3-ketothiolase, or β-oxidation of synthesized fatty acids.

### 2.4.2
### Plants as Polyhydroxyalkanoates Producers

Another important example for establishing PHA biosynthesis is the production by plants. With this strategy, the steps necessary to produce the substrates used in a fermentative process are no longer required, as naturally occurring carbon dioxide and sunlight serve as carbon and energy sources, respectively [6]. In the first investigations reported, the plant *Arabidopsis thaliana*, harboring the PHA genes of *C. necator*, was used to produce P(3HB). An endogenous plant 3-ketothiolase is present in the cytoplasm of this plant as part of the mevalonate pathway. Thus, creation of the P(3HB) biosynthetic pathway in the cytoplasm was theoretically simple, requiring only the expression of two additional enzymes – acetoacyl-CoA reductase and PHA synthase. Today, it is possible to produce P(3HB) and P(3HB-*co*-3HV) utilizing transgenic plants. However, further studies to enhance the PHA percentage and productivity in these plants are necessary [44].

### 2.4.3
### Microbial Degradation of Polyhydroxyalkanoates

Biodegradation is a natural process, mediated by microorganisms that, through their enzymes, can hydrolyze the macromolecules, altering the structure of the material. In all processes of degradation, hydrolysis or hydrolytic degradation is the initial step of the process [87]. Products from the degradation should not be ecotoxic or harmful to the environment. When biodegradation occurs in an aerobic environment, the products formed are usually carbon dioxide, water, organic material, and biomass, while in an anaerobic one, methane and water are generated.

A remarkable characteristic of PHA is its biodegradability in various environments where a considerable number of microorganisms excrete PHA depolymerases to hydrolyze solid PHA into water-soluble oligomers and monomers, which are also utilized as nutrients for their own cells [88].

PHA is degraded in various environments such as soil, sewage, sea water, and lakes. Biodegradation depends on several factors such as microbial activity in the environment, humidity, temperature, pH, and molecular weight of the polymer [89]. The most important factors related to the PHA characteristics which influence its degradation are (i) stereospecificity, since only monomers in configuration (*R*) are hydrolyzed by depolymerases; (ii) crystallinity, since the degradation decreases with higher crystallinity; (iii) molecular weight, because the polymers of low molecular weight are generally degraded more rapidly than those with high molecular weight; and (iv) monomeric composition of PHA [90].

Schneider and coworkers [87] performed a study to determine the biodegradation of P(3HB) films produced from *C. necator* with different oleic acid concentrations (0, 0.3, 0.9, 1.5, and 3.0 $g\,L^{-1}$) as a nutritional supplement for the culture. The biodegradation process was carried out in 5 × 5 cm P(3HB) films, which were buried in beakers in 17-cm-deep soil for 0, 7, 14, and 21 days. The soil humidity was kept between 20% and 30%, based on the dry weight of soil. Figure 2.3 shows the biodegradation of P(3HB) films shown in the Schneider *et al.* [87] study.

According to Figure 2.3, the sample obtained without oleic acid was only slightly decomposed during the studied period (21 days), and the films with 0.3 and 0.9 $g\,L^{-1}$ concentrations showed advanced destruction after 14 days, and were completely destroyed a week later. The films synthesized with 1.5 and 3.0 $g\,L^{-1}$ of oleic acid showed an advanced degradation stage after only 7 days and, after 14 days, it was not possible to find fragments of these samples anymore. These results indicate that kinetic biodegradation of the films in soil was faster with the increase in oleic acid concentration.

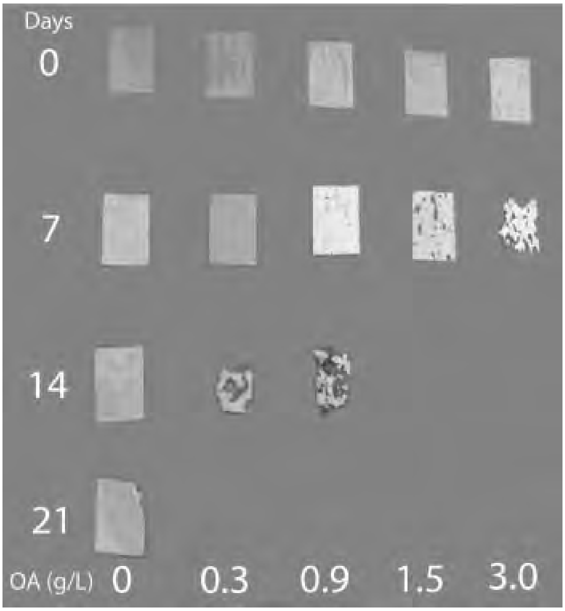

**Figure 2.3** Visual analysis of P(3HB) with different oleic acid content after biodegradation in soil during 0, 7, 14, and 21 days(Ref. [88]).

## 2.5
## Extraction and Recovery

It is estimated that PHA recovery can represent about 50% of the total production cost depending on variables such as the separation process and PHA content in the biomass (%) [91]. As PHA is an intracellular product, the methods adopted for its recovery focus either on its solubilization or on the solubilization of the non-PHA biomass (total biomass excluding PHA) [92]. Figure 2.4 shows methods for PHA extraction.

According to Figure 2.4, the pretreatment aims at the separation of the mixture biomass-fermented medium and can be realized by a mechanical process like centrifugation or a chemical process similar to flocculation either by acidification with sulfuric or phosphoric acids or by adding an alkalizing agent such as calcium hydroxide. One of the processes for PHA isolation is the cell lysis by chemical digestion, which occurs by the use of surfactant and chelate agents, such as anionic sodium dodecyl sulfate (SDS) [93] or synthetic palmitol carnitine [94], sodium hypochlorite, or sodium hypochlorite plus chloroform [95–97]. The enzymatic destruction of cell-wall structures is an effective method to obtain PHA and has been used to achieve lysis of Gram-negative bacteria [98]. Enzymatic destruction of cell-wall structures by less expensive enzymes like pepsin, trypsin, bromelain, papain, and other proteolytic enzymes in order to obtain PHA has been extensively

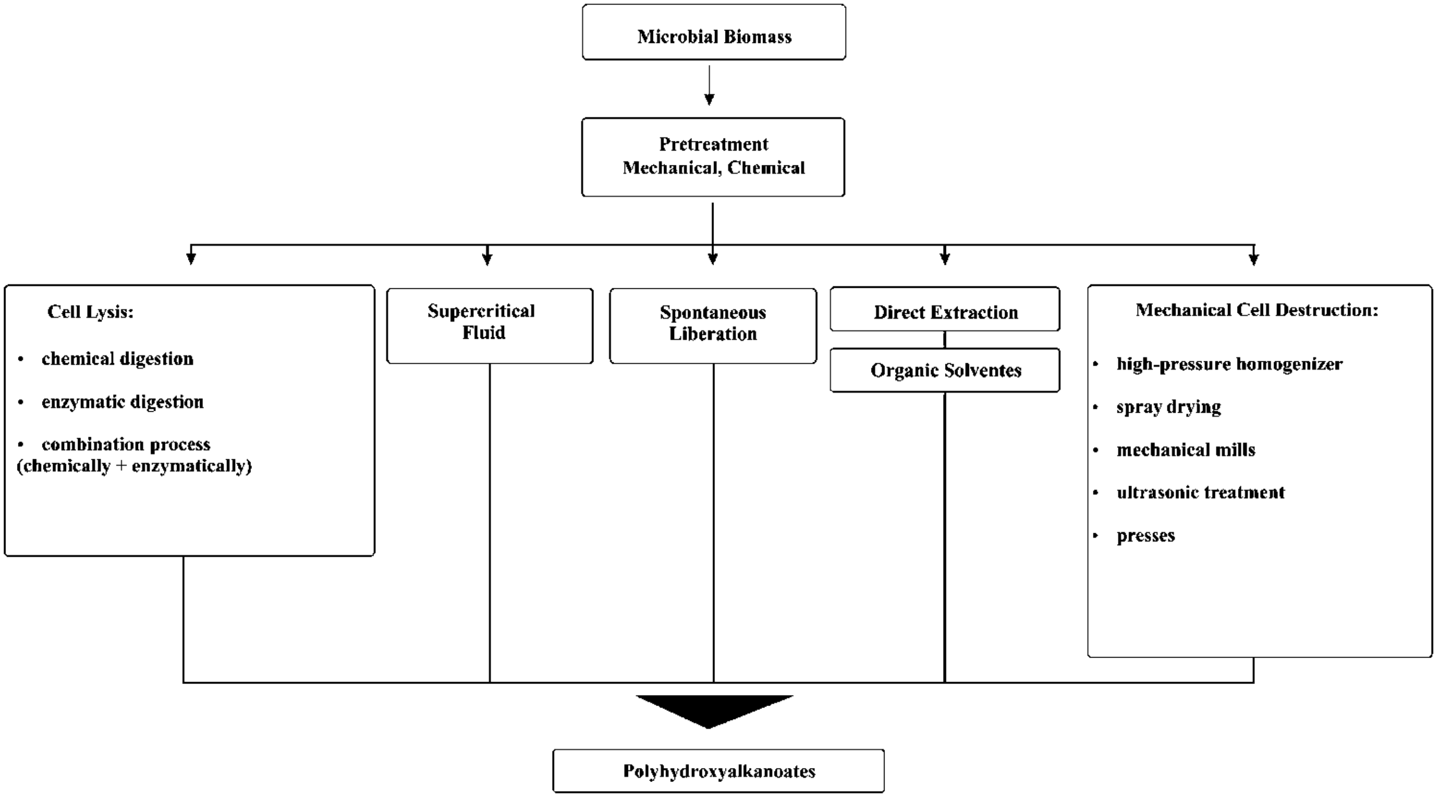

**Figure 2.4** Methods for PHA extraction.

investigated [92, 99, 100]. The use of high pressure in continuously operating cell homogenizers is a usual downstream operation in biotechnology [101, 102]. Direct extractions with organic solvents, chloroform, have been realized by Dalcanton [73]. In this process, cells from *C. necator* cultures were centrifuged and after that they were submitted to extraction with chloroform for 2 h at 60 °C. The ratio of chloroform and biomass was 100 mL and 2 g containing 75% P(3HB), respectively. The results obtained for purity percentage and recovery percentage were 98 and 94, respectively. Other solvents can be used for PHA isolation, whether or not associated with another process, like dichloromethane, pyridine or dichloromethane/ethanol mixtures [31, 32], 1,2-propanodiol, glycerol formal [103], and propylene carbonate [104–106]. Nowadays, genetically modified cells, which have spontaneous liberation of intracellular PHA granules [107], are being studied.

In general, all processes for PHA isolation and purification are expensive, causing a large effluent generation, and it is necessary to develop a cleaner and less expensive process to allow PHA to be competitive in relation to fuel-based polymers.

## 2.6
## Physical, Mechanical, and Thermal Properties of Polyhydroxyalkanoates

Solid-state P(3HB) is a compact right-handed helix with a twofold screw axis (i.e., two monomer units complete one turn of the helix) and a fiber repeat of 0.596 nm [108]. The stereoregularity of P(3HB) makes it a highly crystalline material. It is optically active, with a chiral carbon always in the *R* absolute configuration on biologically produced P(3HB).

The physical properties of PHA are influenced by the molecular weight of the polymer, which depends on the particular bacteria strain used and on the extraction process [109]. The molecular mass distribution of a polymer is a measure of the distribution of its individual molecules' molecular mass around the average molecular mass; a narrow distribution around a high average is usually desired [6].

In addition to being a function of the production organism and the strategy of production (fermentation duration, growth rate, carbon source concentration, etc.), the average molecular mass of PHA is affected by the method of extraction. Values until recently typically ranged from $2 \times 10^5$ to $2 \times 10^6$ Da [5].

Table 2.2 shows a comparison of properties between different PHA, polypropylene, and low-density polypropylene.

According to Table 2.2, P(3HB) and polypropylene show similar melting temperatures ($T_m$), although their chemical properties are completely different. P(3HB) has far lower solvent resistance, specially elongation to break, but it shows a better natural resistance to UV weathering. Physically, P(3HB) is stiffer and brittler than polypropylene [110]. However, when copolymer formation occurs with 3HB and 3HV monomer units, the properties of the material are altered as a consequence of decreased crystallinity and $T_m$. This results, in mechanical terms, in a decrease

**Table 2.2** Comparison of PHA polymers' and common plastics' properties.

| Sample | Melting temperature (°C) | Glass-transition temperature (°C) | Young's modulus (GPa) | Tensile strength (MPa) | Elongation to break (%) |
|---|---|---|---|---|---|
| P(3HB) | 180 | 4 | 3.5 | 40 | 5 |
| P(3HB-*co*-20 mol% 3HV) | 145 | −1 | 0.8 | 20 | 50 |
| P(3HB-*co*-6 mol% 3HV) | 133 | −8 | 0.2 | 17 | 680 |
| Polypropylene | 176 | −10 | 1.7 | 38 | 400 |
| Low-density polyethylene | 130 | −30 | 0.2 | 10 | 620 |

Source: [81].

in stiffness (Young's modulus) and an increase in elongation to break, producing more desirable properties for commercial application [5].

It has been widely accepted that $PHA_{MCL}$ shows amorphous and elastic properties with a lower melting point ($T_m$) and a lower degree of crystallinity compared with short-chain-length PHA ($PHA_{SCL}$) [110, 111]. Interestingly, studies demonstrated that the presence of over 30% long chain comonomer units such as HDD (hydroxydodecanoate−$C_{12}$) and HTD (hydroxytetradecanoate−$C_{14}$) in the $PHA_{MCL}$ increased the $T_m$ and the degree of crystallinity of $PHA_{MCL}$, leading to dramatic changes in $PHA_{MCL}$ mechanical properties [111, 112] making them significantly different from those of the typical $PHA_{MCL}$ [113].

## 2.7
## Future Directions

PHA has quickly gained interest in both research and industry. Their structural versatility and characteristics have been investigated and new areas of exploitation are being discovered. The major drawback for extensive use of these polymers is their high production cost [114]. In this regard, research is continuing on their production from cheap raw materials [71, 72].

Decades of study have been dedicated to the composition and material properties of PHA in this crystallized form. A new perspective for industrial applications of PHA could be the use of native, isolated PHA granules as nano/microbeads in biotechnology and medicine. The PHA granules exhibit all important features of core–shell nanoparticles; their surface has been demonstrated to be easily modified and activity of surface-exposed proteins of interest could be shown [24, 83].

The recent Food and Drug Administration (FDA) approval for the clinical application of P(4HB) suggests a promising future for PHA [114]. The application of PHA in composites scaffolds showing potential for drug delivery has been developed for bone tissue engineering [115]. The emphasis is on the area of medical/biomedical applications including the development to regenerate bone tissue by combining PHA with bone marrow mesenchymal stem cells [116], neural stem cells nanofiber scaffolds [117], among others.

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