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Preparation of novel mcl-PHA by green synthesis: influence of active small molecule via esterification effect on side chain

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Abstract

Microbial production of polyhydroxyalkanoic acid (PHA) with customized features has been attempted by humans through metabolic engineering. However, few studies have explored the green chemical synthesis of customized PHA using hydroxy fatty acids. In the present study, new mcl-PHA having C₁₆–co-C₁₆, blocked by esterification of hydroxy groups in the side chain, was synthesized by melt polycondensation using aleuritic acid (9, 10, 16-trihydroxyhexadecanoic acid) and lactic acid as long-chain fatty acid monomers and active blocking molecule, respectively. The results showed that when the two monomers reacted at 150 °C for 24 h, the properties of PHA were largely retained, along with free hydroxyl groups as much as possible. Comparison of the effect of different ratios of active small molecule (lactic acid) on the properties of new mcl-PHA revealed that copolymerization mainly happened between the long-chain monomers, and lactic acid served as a blocker by esterification in the side chain on the hydroxy groups of mcl-PHA. Further, the properties of side chain-esterified mcl-PHA (SCE-mcl-PHA) changed from rigid to soft with increasing ratio of active lactic acid monomer. The tensile strength of mcl-PHA was similar to that of commercially available products PLA and PHBV. The mechanical properties of SCE-mcl-PHA significantly changed by adding only 5 wt% lactic acid monomer. Besides, the cell proliferation analysis showed that the new PHA had minimum cytotoxicity, which was close to PLA and PHBV. Therefore, these new mcl-PHA and SCE-mcl-PHA have great potential for application in food, medicine, cosmetics, and other fields, and hence require further investigation.

Introduction

Polyhydroxyalkanoic acid (PHA) is the general term for a family of macromolecular compounds formed through polymerization of hydroxy fatty acids via ester bonds [1, 2]. PHAs are considered to be an ideal substitute for plastics from fossil fuels in the future due to their good quality and biodegradability [3–5]. They are primarily produced by engineering microbial metabolism in humans nowadays. This area has been constantly enriched by various studies that emerged and continue to emerge in recent years on the basis of microbial–metabolic reactions for PHA production [6–10]. However, sufficient support for the microbial production of PHAs cannot be achieved constantly with the types of monomers, polyester structure, and physical and chemical properties under various circumstances pertaining to microbial species, carbon sources, culture conditions, and other factors [10–13]. At least, they cannot meet certain specific requirements for sophisticated properties of the materials as petroleum-based plastics do during actual production [14–16]. Hence, efficient ways to produce PHAs with fantastic properties need to be urgently explored. For this purpose, many previous studies have
investigated the chemical modification of PHAs and the regulation of microbial metabolism [17–19]. Benítez and colleagues performed polymerization of aleuritic acid by noncatalyzed melt condensation and explored the modifications by addition of palmitic acid and oxygen on the air-exposed side [20–22]. They proved that it was feasible to prepare PHAs by chemical synthesis method. Variations in microbial species and the types and structure of PHA monomers in biosynthesis are limited, and the synthesis of long-chain monomers is more difficult and costly [23–26]. However, the nature of PHA materials is determined by their composition, proportion, and distribution [27–29]. Large-scale preparation of PHAs by engineering microbial metabolism is driven to a large extent by the continuous renewal and growth of its long-chain monomers [30–33]. Taken together, the introduction of long-chain monomers brings about a variety of optional variables such as types of side chains, functional groups, and number of ester bonds per chain unit, providing greater possibilities for enhancing and improving the properties of PHA.

The advantages of microbial-derived PHAs are their good biodegradability, biocompatibility, and renewability [34,35]. The corresponding advantage of using microbial metabolism for preparing PHAs lies in its environmental protection and sustainability [36–38]. Therefore, the biodegradability, biocompatibility, and renewability of natural PHAs can be achieved when the natural source of hydroxy fatty acids is used as monomers in the chemical synthesis of PHA. If PHAs are prepared by a clean and environment-friendly method, the number of options for the monomer selection may increase, which would be as advantageous as microbial-derived PHAs despite their production by synthetic methods. The melt polycondensation has been proved to be a green method due to its noncatalyzed and solvent-free attribute. Using this method, a new PHA with aleuritic acid as polymeric monomer could be prepared and a small molecule was introduced to improve the hydrophilicity or mechanical properties. Hence, two kinds of easily available and sustainable hydroxyl fatty acids, aleuritic acid (C16) and lactic acid (C3), were chosen for this study, which were isolated from natural resin-based materials (lac resin) of insects as polymerization monomer and active small molecule, respectively. Then, a novel medium-chain-length PHA (mcl-PHA) was prepared via melt polycondensation. The influence of reactive conditions on the properties of PHA was revealed by structure and performance characterization of PHA. The present study provided the way to prepare a new PHA, containing hydroxy functional group, from specific monomers by chemical synthesis and a new train of thoughts for the chemical preparation of aleuritic acid based PHA which owned both excellent mechanical properties and hydrophilicity by hydroxyl protection method in the future.

**Experiment**

**Materials and reagent**

Aleuritic acid (purity ≥95%, HPLC) was prepared by the Research Center of Engineering and Technology on Characteristic Forest Resources, State Forestry Administration, Research Institute of Resources Insects, and Chinese Academy of Forestry. Lactic acid (≥85%, titration) was purchased from Aladdin Co. Ltd (Shanghai, China). Polylactic acid (PLA) was purchased from Shenzhen Esun New Materials Co. Ltd (molecular weight ≈ 1.4 × 10^5). Poly(3-hydroxybutyrate-co-3-hydroxyvalerate (PHBV) was purchased from TianAn Biologic Materials Co., Ltd (Ningbo, China).

**Preparations for chemosynthetic PHA(CS-PHA) via melt polycondensation**

First, the proportion of aleuritic acid and lactic acid monomers was accurately controlled at a mass ratio. They were mixed in a round-bottomed flask and heated to 110 °C in a constant-temperature metal bath with magnetic stirring. After melting of the samples, they were transferred to a polytetrafluoroethylene mold (length × width × depth = 125 × 5 × 3 mm [3]). The chemical reactions took place in a constant-temperature reaction chamber at 150 °C [21, 22]).

**Reaction time of preparations for CS-PHA via melt polycondensation**

In the aforementioned method, the mass ratio of 9:1 between aleuritic acid and lactic acid was selected as an appropriate proportion to confirm the reaction time by melt polycondensation. The test samples were collected at 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, and 24 h after the initiation using attenuated total reflectance Fourier transform infrared spectroscopy, differential scanning calorimetry, thermogravimetric (TG) analysis, x-ray photoelectron spectroscopy (XPS) analysis, and contact angle measurements.

**Effects of monomer ratio on the properties of CS-PHA**

Accurately weighed aleuritic acid and lactic acid monomers were kept at a mass ratio of 10:0, 9.5:0.5, 9:1, 8.5:1.5, and 8:2. In other words, the ratio of lactic acid was 0% (control), 5%, 10%, 15%, and 20%, respectively. After 24-h reaction, the rubber strips of PHA were sampled for further examination using differential scanning
calorimetry, thermogravimetric analysis, dynamic thermal mechanical analysis (DMA), x-ray diffraction analysis, contact angle measurements, and cell culture cytotoxicity assay.

Characterizations

**Attenuated total reflectance (ATR)–Fourier transform infrared (FTIR) spectroscopy**

PHAs were sampled in moderate quantities and repeatedly scanned 32 times using ATR–FTIR spectroscopy (Tensor-27, Brucker, Germany). ATR module was used in the range of 4000–400 cm\(^{-1}\) wavenumber with a resolution of 4 cm\(^{-1}\).

**Differential scanning calorimetry (DSC)**

Accurately weighed aliquots of PHA samples (5 mg) were scanned by DSC (200F3, Netzsch, Germany) in the temperature range of 0 °C–550 °C and –80 °C to 550 °C at a heating rate of 10 °C min\(^{-1}\). The crucible was made of aluminum, and the flow rate of purging gas and protective gas (nitrogen) was 80 ml min\(^{-1}\) and 50 ml min\(^{-1}\), respectively.

**Thermogravimetric analysis (TG)**

The prepared PHA samples were collected with weighed aliquots of 5–8 mg and scanned by TG (STA2500, Netzsch) in the temperature range of 30 °C–800 °C at a heating rate of 10 °C min\(^{-1}\). The crucible was made of alumina ceramic, and the flow rate of purging gas and protective gas (nitrogen) was 100 ml min\(^{-1}\) and 80 ml min\(^{-1}\), respectively.

**XPS analysis**

The PHA-derived rubber strips were trimmed into circular membranes of approximately 5-mm diameter. Imaging x-ray photoelectron spectroscopy (XPS, PHI5000 Versaprobe-II, Ulvac-Phi, Japan) and scanning electron microscopy were used at a voltage of 15 kV for a 50 W input power. For the anode Al target, the reference energy peak of Cls was corrected to 284.8 eV, and the value for pass energy was 46.95 eV.

**X-ray diffraction analysis**

The samples were tested in the reflection mode using x-ray diffractometer (D/max-2200, Rigaku, Japan) as follows: Cu Kα wavelength (\(\lambda = 1.5405 \times 10^{-10}\) m); voltage, 40 kV; current, 40 mA; and scan range, 2\(\theta = 5^\circ–90^\circ\) at a scanning rate of 8 °/min. The crystallinity ratio was calculated using MDI Jade 6.5 analysis software by the following equation:

\[
X_c = \frac{I_c}{I_c + I_a} \times 100\%
\]

where \(X_c\) is the crystallinity of CS-PHA; \(I_c\), intensity of crystal peak; and \(I_a\), intensity of amorphous peak.

**Stress–strain measurement during the tensile test**

Samples of PHA were cut into rubber strips of approximately 50 × 5 × 1 mm\(^3\) (length × breadth × thickness). The stretching rate was measured using a universal testing machine (UTM6000, SUNS, China) at the rate of 5 mm min\(^{-1}\).

**DMA**

The PHA samples were trimmed into strips of approximately 50 × 5 × 1 mm\(^3\) (length × breadth × thickness) for DMA (DMA242C, Netzsch) test using dynamic rheometer with a tensile clamp. Dynamic scanning was performed in the temperature range of –80 °C to 200 °C at a heating rate of 10 °C min\(^{-1}\).

**Contact angle measurements**

Both PLA and PHBV were produced by the melting method, and the contact angle (SL200KS, Kino, America) was measured by the static method using deionized water at a volume of 2 μl. The photographs were taken once the liquid fell down and stabilized. Each sample was measured at least five times.

**Solid-state nuclear magnetic resonance (SSNMR)**

Solid-state nuclear magnetic resonance spectroscopy NMR analysis were performed on 500 MHz Advance III (Bruker) SSNMR spectrometers using a 4-mm MAS probe, ZrO\(_2\) rotor with MAS spin rate at 10000 Hz, contact time 2 ms. Typical radiofrequency (rf) field strength was 40–50 kHz for \(^{13}\)C. The chemical shifts were externally referenced to the adamantane CH\(_2\) signal at 38.48 ppm on the TMS scale.
To evaluate the cytotoxicity of CS-PHA with living cells, mouse mesenchymal stem cells (mMSCs) (Procell Life Science & Technology Co., Ltd, China) were used to seed on the samples. The CS-PHA and its reference materials for testing were prepared as follows: PLA and PHBV were dissolved in chloroform, and then the solution was pipetted into a Teflon circular mold (60 mm diameter × 1 mm thickness). The reference control films were obtained after drying for 3 days at room temperature and pressure. The CS-PHA films (0%, 5%, 10%, 15%, and 20% lactic acid) were prepared by blending two monomers into a circular mold (60 mm diameter × 1 mm thickness) at 150 °C for 24 h. Then, perforators were used to trim all the prepared films into samples of 3 mm diameter. Using this method, circular membranes having the following compositions were prepared: PLA, PHBV, and CS-PHA (0%, 5%, 10%, 15%, and 20%). All samples were sterilized at 121 °C for 30 min before cell culture cytotoxicity experiment. Minimum Eagle’s medium (MEMα, purchased from Thermo Fisher Scientific, China) was chosen as the growth medium, and 10% fetal calf serum (FBS) and 1% antibiotics (composed of streptomycin and penicillin) were supplemented in the growth medium. All samples were subdivided into subgroups as follows: (1) blank controls: culture medium only (MEMα); (2) normal controls: cell culture medium (MEMα) with mMSCs; and (3) samples: culture medium (MEMα), mMSCs, and testing samples (PHAs). The cell culture was performed as follows. Well-growing mMSCs in a logarithmic growth stage were resuspended, and then their density was adjusted to 5 × 10^3 cells/200 μl in the MEMα medium. The samples (circular membranes) were transferred into a 96-well plate in triplicates. Next, 200 μl of cell suspension was pipetted onto each sample. Phosphate-buffered saline was used to fill empty wells. After 48-h incubation at 37 °C, all wells were supplemented with 20 μl MTT and cultured at 37 °C for another 4 h. The medium was collected and then mixed with 200 μl DMSO per well, followed by shaking for 10 min. Finally, the mixtures of each well were moved into a new 96-well plate, and the absorbance of each well at 568 nm wavelength was determined using a microplate reader.

Results and discussion

Reaction time of preparations for CS-PHA via melt polycondensation

The melting peak of the low-melting-point zone gradually moved toward a lower-temperature range as the reaction proceeded (figure 1). The melting peak of the samples decreased from 82.3 °C (eutectic temperature between aleuritic acid and lactic acid [42], 0 h) to 44.2 °C (6 h) thereafter and disappeared completely after 7 h. Collectively, the melting peak disappeared following 7 h polymerization of aleuritic acid with lactic acid, indicating that 0–7 h was the basic time requirement for polymerization from all monomers. However, whether reactions for CS-PHA samples can be completed within 7 h remains unanswered. Basically, it can be referred to the effects of melt processing on the samples in the high-temperature zone. Two distinct endothermic peaks, 371.7 °C and 429.3 °C, appeared throughout the high-temperature range, which were presumably the vaporization or decomposition temperatures of the aleuritic acid monomers (figure S1 is available online at stacks.iop.org/MRX/6/075328/mmedia). Comparatively, the endothermic peak around 459.8 °C was the decomposition temperature of the newly formed CS-PHA polymers. More importantly, it gradually emerged after 9 h and then became more prominent as the reaction proceeded. These results indicated that the

Figure 1. Alterations in the DSC curve of CS-PHA over time.
polymerization of the free monomers in the samples mainly occurred within 9 h, which was basically regarded as the prepolymerization of PHA. Therefore, the reaction between two monomers by condensation polymerization needed at least 10 h.

As shown in Figure 2, the absorption peaks of carbonyl vibration in aleuritic acid and lactic acid were located around 1723 cm\(^{-1}\), while in the following reactions, the absorption peak of blue-shifted C=O stretching vibration of carbonyl group moved toward 1737 cm\(^{-1}\), indicating the formation of ester bonds. As the reaction proceeded, a new absorption peak of polyester carbonyl group (C=O) appeared in 1634 cm\(^{-1}\), and the absorption intensity obviously increased over time. Further, 1460 cm\(^{-1}\) was an absorption peak of deformation vibration of carboxyl COOH, where the absorption peak significantly decreased with the strength of the reaction, also indicating that the esterification reaction continued to proceed\(^{[20]}\). According to infrared spectroscopy, the absorption peak of vibration for carboxyl group around 1460 cm\(^{-1}\) gradually weakened from 0 to 24 h, suggesting that the polymerization of the two monomers lasted for at least 24 h at 150 °C. Obviously, the polymerization continued to proceed over time, but its reaction rate rapidly reduced, so did its reaction intensity. Therefore, the question is how to control the correlation between reaction intensity and CS-PHA properties (the extent of polymerization). For example, although an increase in the degree of cross-linking might enhance the strength of the material, it was likely to result in a decrease in toughness and a series of changes, such as the decrease in the number of hydroxyl groups and the reduced hydrophilicity, which could be well demonstrated by alterations in absorption peak in the infrared fingerprint region. A new absorption peak of C–O–C stretching vibration of polyester appeared around 1185 cm\(^{-1}\), which also showed a changing process after emergence, from weak to strong, over time. Moreover, 1135 cm\(^{-1}\) and 1058 cm\(^{-1}\) were characteristic absorption peaks of primary hydroxyl –CH\(_2\)OH and secondary hydroxyl –CHOH, respectively, and both absorption peaks gradually weakened over time, suggesting the reaction of monomers or esterification on the side chain of long-chain monomer. Therefore, the reaction time must be precisely controlled to well manage the relationship between the strength of PHAs and their properties.

The PHA samples were collected for XPS C1 analyses 0, 7, 12, 24, and 48 h after initiation, followed by calculations and data simulation using the Multipack 9.3 software (figure 3). The energy peak of C–C/C–H binding was 284.8 eV. The XPS C1 spectra of PHA showed a trend similar to those of DSC and FTIR over time (table 1). For the functional group of C–O–H (287.8 eV)\(^{[43]}\), for instance, the mass ratio of aleuritic acid to lactic acid was 9:1, and the ideal molar ratio was about 2.66:1, regardless of impurities in raw materials. Moreover, the molar ratio of hydroxyl group to carboxyl group was about 2.45:1, as being equivalent to monomer composition. The hydroxyl groups were obviously excessive in the composition of all functional groups during polymerization between lactic acid and aleuritic acid. Hence, two of the important explorations in the synthesis of PHA via melt polycondensation in this study were to retain as many active groups (hydroxyl groups) as possible in the original structure and explore the relationship between the extent of its retention for active groups and their properties.

According to the correlation between the content of C–O–H and the polymerization processes over time, the decreases in the contents of hydroxyl groups are shown in table 1. The content of C–O–H reduced with the polymerization reaction. Two main types of reaction occurred. One was polymerization reaction between monomers, and the other was dehydration reaction of the C–O–H from aleuritic acid. DSC and FTIR results

Figure 2. Alterations in FTIR of CS-PHA over time.
Figure 3. XPS C1 analysis of CS-PHA over time: (a) 0 h, (b) 7 h, (c) 12 h, (d) 24 h, and (e) 48 h.

Table 1. XPS C1 analysis of CS-PHA over time at different time points: (a) 0 h, (b) 7 h, (c) 12 h, (d) 24 h, and (e) 48 h.

| Group | C–C, C–H | C’–O–C=O | C–O–H | C–O–C’=O |
|-------|-----------|-----------|--------|-----------|
| Chemical shift/eV | 284.8 | 286.4 | 287.8 | 289.0 |
| 0 h | 80.59 | 12.26 | 1.58 | 5.56 |
| 7 h | 79.38 | 13.84 | 1.34 | 5.48 |
| 12 h | 79.18 | 13.94 | 1.29 | 5.67 |
| 24 h | 79.95 | 13.13 | 1.18 | 5.74 |
| 48 h | 82.04 | 11.28 | 0.82 | 5.86 |
showed that the polymerization reaction could last for 24 h. After that, the extension of reaction time had no effect on the polymerization of monomers. On the contrary, it accelerated the dehydration reaction, and the color of CS-PHA changed from shallow to deep (figure S2) with an increase in the contact angle of CS-PHA (figure 4 and table 2). Considering the extent of the reaction and the effect of changing the monomer ratio on the properties of CS-PHA, follow-up tests were performed after the 24 h reaction.

Effect of ratio of short-chain monomer on the properties of CS-PHA

Because of the crystallinity of aleuritic acid (figure S3), the emergence of amorphous structure (figure 5(a)) and the dramatic decrease in crystallinity (relative to aleuritic acid, figure 5(b)) also suggested the formation of polymers and the occurrence of polymerization reactions. The DSC curve (figure 6(a)) showed that the glass transition temperature of CS-PHA decreased significantly with the increase in the ratio of short-chain monomer lactic acid (table 3), which was consistent with the results of DMA measurements (figure 7(b)). Although the glass transition temperature slightly decreased, it was obviously not consistent with the conventional view that the glass transition temperature of PHAs formed by 3-hydroxy fatty acids decreased with the increase in the monomer number of long-carbon chain polymers [44]. A reasonable explanation was that the glass transition temperature of the traditional 3-Hydroxy PHAs were susceptible to the alterations in side chain groups. A larger number of carbon atoms and longer side chain groups led to a lower energy or temperature required for the free movement of the molecular carbon chain in 3-hydroxy PHA, thereby achieving a lower glass transition temperature pertaining to the properties. The CS-PHA composed of lactic acid and aleuritic acid was able to form a linear polyester without side chains. Taking a step back, despite interaction between hydroxyl groups in the aleuritic acid monomer at 9, 10 position of each monomeric unit, the existence of free hydroxyl groups in the side chains made it easier to form hydrogen bonds between the side chains or between the side chains and the main chains. Therefore, the binding forces of the free movements of the side chains were further enhanced. Thus, different from the traditional 3-hydroxy PHAs, the glass transition temperature of CS-PHA was primarily determined by the functional groups of the monomer itself and the structure of the main chain. The possible reason was that the molecular structure of mcl-PHA was altered with the addition of lactic acid, especially the cross-linking or intermolecular force between free hydroxyl groups. This was also supported by the decreases in the temperature of endothermic peaks (300 °C–400 °C, figure 6(a)) from the DSC curve and decrease in the initial temperature of thermogravimetric analysis (figure 6(b)) for CS-PHA from the TG curve. The decrease in temperature pertaining to CS-PHA production was due to the increase in the number of ester bonds, which was caused by an increase in the number of short-chain monomers along the polymeric chains. However, the question was whether the lactic acid participated in the copolymerization. The determination of mechanical properties could possibly give directional results.

The polymer of aleuritic acid (0%, mcl-PHA) had a slightly higher tensile strength and strain range than those of the two commercial forms of PHA (PLA and PHBV) (figure 7(a)), reaching 8.65 MPa and 195.9%
With the introduction of lactic acid comprising short-chain monomers, the stress and strain decreased rapidly. When the proportion of lactic acid monomer was 15%, the tensile strength of CS-PHA reduced to 0.15 MPa, indicating that the original polymerization mode or intensity of aleuritic acid was drastically altered due to the introduction and increase in short-chain monomers. The fracture of the autopolymer made of aleuritic acid was more like a 'flexible' fracture, while the fracture of CS-PHA was closer to a 'brittle' fracture. Figure S4 shows the PHA samples that had undergone these two fractures. The introduction of short-chain monomers not only reduced the mechanical strength but also brought alterations in toughness. The results uncovered the effect of the increasing ratio of lactic acid (0%, 5%, 10%, 15%, and 20%) on the mechanical properties of PHA (figure 8). Hence, the CS-PHA–derived stripes exhibited a remarkable transition from rigid to soft type with the increase in the ratio of lactic acid. The number of coils in the figure represents the degree of flexibility in the stripes. Furthermore, 10% of lactic acid content became its critical point. In particular, when the content of lactic acid was less than 10%, PHA exhibited more toughness and mechanical strength. In contrast, when the content of lactic acid was higher than 10%, PHA became softer with less mechanical strength.

This study also clearly demonstrated the association of the contact angle for PHA with the increase in short-chain monomer lactic acid (figure 9). Both Ale-co-Ale PHA and LA-co-Ale-PHA, which were made of long-chain and short-chain lactic acid monomers, exhibited better hydrophilicity than that of control PHA made of two short-chain monomers (table 5). The contact angle of mcl-PHA using aleuritic acid as the monomer was only 68.5°. Nevertheless, the hydrophilicity of CS-PHA increased with an increase in lactic acid content. Two possible reasons for this were as follows. First, compared with short-chain monomer PHA, long-chain monomer PHA reduced the number of ester bonds in the polymer even with the same molecular weight, thus making it easier to achieve better hydrophilicity [45]. Second, the aleuritic acid used in this study was 9, 10, 16-trihydroxy hexadecanoic acid. Hence, the hydrophilicity of CS-PHA was enhanced with an increase in the number of hydroxyl groups on the polyester chain [46].
Special characteristics were determined from the structures. Thus, alterations in mechanical energy in mcl-PHA or CS-PHA were primarily due to the introduction of a short-chain lactic acid monomer, resulting in a structural change in CS-PHA. Based on existing evidence, two conjectures were put forward. First, lactic acid monomers were introduced into the main chain structure of CS-PHA. Apparently, it did not accord with the rapid reduction in mechanical properties with the addition of lactic acid (figure 7). Second, lactic acid monomers were not introduced or not completely introduced into the main chain structure of CS-PHA (Scheme 1). The differences in the properties of CS-PHA were due to the alterations in polymerization mainly mediated by lactic acid instead of aleuritic acid. In this case, lactic acid acted like a ‘blocker’ during the polymerization of aleuritic acid because both autopolymers exhibited better structural strength (table 4) regardless of the monomer choice, namely, lactic acid and aleuritic acid (PLA and 0% CS-PHA).

The introduction of lactic acid monomer might have a blocking effect on the linear polymerization of aleuritic acid, preventing further polymerization of aleuritic acid monomer or cross-linking between PHA chains. Figure 10 shows that the polyester was generated successfully (figure 10(b)), and abundant

**Table 3. Analyses for thermal properties of reference substance PHA and CS-PHA.**

| Monomer | PLA | PHBV | 0% | 5% | 10% | 15% | 20% |
|---------|-----|------|----|----|-----|-----|-----|
| $T_g$/DSC $^a$ | — | — | −11.69 | −12.73 | −13.15 | −13.30 | −13.70 |
| $T_g$/DMA $^b$ | −11.83 | −12.31 | −11.85 | −12.35 | −13.58 | −14.23 | −12.77 |

$^a$ is measured by DSC.

$^b$ is measured by DMA.

![Figure 6. Correlation between thermal properties of CS-PHA and its monomer ratio.](image-url)
ortho-dihydroxy groups (figures 10(a) and (c)) were retained in the mcl-PHA from aleuritic acid, as reported by Benítez [21]. With the addition of lactic acid, the signal peak of polyester faded gradually and the signal peak of esterified ortho-dihydroxy groups (figure 10(d)) enhanced relatively. The results indicated that the polymerization occurred between aleuritic acid monomers and weakened when lactic acid was introduced into the reaction system. Obviously, of the two aforementioned speculations, the second one was more reasonable.

**Table 4.** Analyses for mechanical properties of reference substances PHA and CS-PHA.

|                | PLA  | PHBV| 0%  | 5%  | 10% | 15% | 20%  |
|----------------|------|-----|-----|-----|-----|-----|------|
| Stress(MPa)    | 8.06 | 8.47| 8.65| 1.39| 0.37| 0.15| 0.24 |
| Strain(%)      | 159.9| 180.6|195.9| 78.51| 35.0| 26.3| 26.7 |
| Young’s modulus(MPa) | 17.73| 1.73| 9.45| 8.16| 1.32| 0.82| 1.11 |

**Figure 7.** Stress–strain measurements and DMA curves of reference substances PHA and CS-PHA.

**Figure 8.** (a) 0% LA-PHA/mcl-PHA, (b) 5% LA-PHA, (c) 10% LA-PHA, (d) 15% LA-PHA, and (e) 20% LA-PHA.
The CS-PHA obtained by the second reaction path was called side chain–esterified mcl-PHA (SCE-mcl-PHA). Because no solvent was suitable for dissolving this new SCE-mcl-PHA, its molecular weight has not been accurately measured yet. In any case, it does not negate the significance of the synthesis of this new mcl-PHA and SCE-mcl-PHA in this study because it can be directly applied to food packaging [47] and biomedical materials [48], which do not require much strength, following further modifications of its free hydroxyl groups as the active sites. Therefore, its toxicological effects need further investigation immediately.

The MTT assay was used to detect the cytotoxicity of the PHA material (figure 11). The CS-PHA made of aleuritic acid and lactic acid through melt polycondensation obviously promoted the growth and proliferation of mMSCs compared with the cells from other control groups when the percentage of a small molecule, lactic acid, was less than 10%. It has been proved that the cells adhere and grow more easily on the moderately hydrophilic materials than on hydrophobic surfaces [14, 49, 50]. Therefore, the variability in cytotoxicity in the present study might correlate with the esterification degree of hydroxyl groups in the side chain and the hydrophilicity of CS-PHA. Although the effect of a chemical structure should not be neglected, the cytotoxicity of CS-PHA was possibly related to the contact angle (figure 9) to a certain extent. Why some CS-PHAs promoted the proliferation has not been completely explained yet [51–52]. Obviously, the cytotoxicity of PHA materials was highly relevant to many factors such as the monomer compositions, molecular structures and molecular

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**Table 5.** Correlation between the contact angle of reference substances PHA/CS-PHA and lactic acid content.

| Samples                  | Contact angle (degree) |
|--------------------------|------------------------|
| PLA (melted film)        | 78.0 ± 1.1             |
| PHBV (melted film)       | 80.0 ± 1.6             |
| 0%LA-PHA                 | 68.5 ± 0.4             |
| 5%LA-PHA                 | 71.0 ± 0.7             |
| 10%LA-PHA                | 76.5 ± 0.8             |
| 15%LA-PHA                | 72.5 ± 2.2             |
| 20%LA-PHA                | 79.5 ± 1.5             |

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**Scheme 1.** Schematic illustration of the synthetic pathway for CS-PHA and blocking the effect of lactic acid on SCE-mcl-PHA.

**Figure 9.** Water drops on the surface of reference substances PHA and CS-PHA: (a) PLA, (b) PHBV, (c) 0% LA-PHA/mcl-PHA, (d) 5% LA-PHA, (e) 10% LA-PHA, (f) 15% LA-PHA, and (g) 20% LA-PHA.
weights, etc. The findings suggested that CS-PHA samples had no cytotoxicity and were close to PLA and PHBV in terms of cytotoxicity. PLA and PHBV were microbial PHAs and the cytotoxicity evaluation results indicated excellent biocompatibility [14, 53, 54]. Hence, the bio-based source of monomers, aleuritic acid and lactic acid, may be a favorable factor for the cytotoxicity of CS-PHA materials. Nevertheless, cell proliferation was found to be slightly inhibited by CS-PHA with the increase in the ratio of lactic acid. For instance, the relative proliferative rates of cells treated with 15% and 20% CS-PHA reduced from 99.3% to 77.9%. In summary, the results of cytotoxicity of aleuritic acid based mcl-PHA and SCE-mcl-PHA revealed they have enormous potential in using as biocompatible materials in the future.

Conclusions

In the present study, PHA was synthesized by a green method, melt polycondensation, using two kinds of natural hydroxy fatty acids, aleuritic acid and lactic acid, as a source of chemical raw materials. The results showed that a novel SCE-mcl-PHA was obtained via polymerization of monomers at 150 °C. When the polymerization lasted
for 24 h, the monomers reacted completely and the free hydroxyl group of aleuritic acid was conserved as far as possible, thereby improving the hydrophilicity of the new CS-PHA. The mcl-PHA formed by the self-polymerization of aleuritic acid achieved a similar level or even slightly surpassed the level of tensile strength in commercially available PLA and PHBV. However, the evaluation of mechanical properties and SSNMR indicated that the main copolymerization happened between the long-chain monomers, and the introduction of an active small molecule, lactic acid, served as a blocker by esterification of hydroxyl groups in the side chain. Further, increasing the ratio of short-chain lactic acid monomers (5%, 10%, 15%, and 20%) led to the formation of SCE-mcl-PHA, rapidly adjusting their properties from rigid to soft. The properties of SCE-mcl-PHA significantly changed by adding only 5 wt% lactic acid monomer. Fortunately, the results of cell culture showed that both mcl-PHA and SCE-mcl-PHA obtained by melt condensation showed minimum cytotoxicity compared with microbial PHA (PLA and PHBV). This synthetic new type of PHAs not only has great potential for its application in food, medicine, cosmetics, and other fields but also provides deep insights into the modification of PHA using free hydroxyl groups and the production of new synthetic PHA.

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Conflicts of interest

There are no conflicts to declare.

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References

[1] Kumar M, Singhal A, Verma P K and Thakur I S 2017 Ac. Omega. 2 9156
[2] Ming W D X M Y, Hui Y, Lin-Ping W and Qiang C G 2016 Biomacromolecules 17 2680
[3] Almeida E L, Margassery L M, O’Leary N and Dobson A D W 2018 Genome Announcements 6 e01534
[4] Cruz M V, Araújo D, Alves V D, Freitas F and Reis M A M 2016 International Journal of Biological Macromolecules 82 243
[5] Parlane N A, Gupta S K, Reyes P R, Chen S and Rehm B H A 2016 Ac. Biomaterials Science & Engineering. 3 acbibiomaterials.6b00355
[6] Hu D, Chung A-L, Wu L-P, Zhang X, Wu Q, Chen J-C and Chen G-Q 2011 Biomacromolecules 12 3166
[7] Wang Q, Tappel R C, Zhu C and Nomura C T 2012 Applied and Environmental Microbiology 78 519
[8] Wang Y, Chung A and Chen G Q 2017 Adv. Healthcare Mater. 1601017
[9] Witholt B and Kessler B 1999 Current Opinion in Biotechnology 10 279
[10] Yasotha K, Aroua M K, Ramachandran K B and Tan I K P 2006 Biochem. Eng. J. 30 260
[11] Arslan H, Hazer B and Yoon S C 2007 J. Appl. Polym. Sci. 103 81
[12] Bhatt R, Panchal B, Patel K, Sinha V K and Trivedi U 2010 J. Appl. Polym. Sci. 110 975
[13] Cakmakli B, Hazer B and Borcakli M 2001 Macromolecular Bioscience. 1 348
[14] Insomphun C, Chuah J A, Kobayashi S, Fujiki T and Numata K 2016 Ac. Biomater. Sci. Eng. 3 3064
[15] Insomphun C, Kobayashi S, Fujiki T and Numata K 2016 Amb Express. 6 29
[16] Panaitescu D M, Lupescu I, Frone A N, Chianam I, Nicolae C A, Tofan V, Stefaniu A, Somoghi R and Trusca R 2017 Biomacromolecules. 18 3222
[17] Alzate Marin J C, Rivero S, Pinotti A, Caravelli A and Zaitzky N E 2018 Journal of Agricultural and Food Chemistry 66 10033
[18] Ansari N F and Annuar M S M 2017 Journal of Macromolecular Science, Part A. 1 1
[19] Li Z and Loh X J 2015 Cheminform. 46 2865
[20] Benitez J J, Guzmán-Puyol S, Domínguez E and Heredia A 2015 J. Appl. Polym. Sci. 132
[21] Benitez J J, Heredia-Guerrero J A, Cruz-Carrillo M A, Morales-Flórez V, Rosa-Fox N D L and Heredia A 2016 J. Phys. D: Appl. Phys. 49 175601
[22] Benitez J J, Heredia-Guerrero J A, Guzmán-Puyol S, Domínguez E and Heredia A 2015 Soft Materials. 13 5
[23] Blunt W, Dartyjail C, Sparking R, Capes D, Levin D B and Cizek N 2017 Proc. Biochem. 59 18
[24] Davis R, Kataria R, Cerrone F, Woods T, Kenny S T and Babu R P 2014 Acs Biomaterials Science & Engineering. 9 156
[25] Federico C, Choudhari S K, Reeta D, Denise C, Vincent O F, Gearoid D, Eoin C, Guzik M W, Kenny M W, Kenny S T and Babu R P 2014 Applied Microbiology & Biotechnology 98 611
[26] Siracusa V, Roccu P, Romani S and Rosa M D 2008 Trends in Food Science & Technology 19 634
[27] Champanet P S 2010 Journal of Bioscience & Bioengineering 110 621
[28] Gummel A M and Annuar M S M 2013 Journal of Polymers & the Environment 21 580
[29] Reddy C S K, Ghaori R and Kalia V C 2003 Bioresour. Technol. 87 137
[30] Budde C F, Riedel S L, Willis I B, Chokyrun R and Simskey A J 2011 Applied & Environmental Microbiology 77 2847
[31] Gogolewski S, Jovanovic M, Perren S M, Dillon J G and Hughes M K 1993 J. Biomed. Mater. Res. 27 1135
[32] Lu J, Tappel R C and Nomura C T 2009 Polymer Reviews 49 226
[33] Timm A and Steinbüchel A 1990 Applied & Environmental Microbiology 56 3360
[34] Hiroe A, Ishii N, Ishii D, Kabe T, Abe H, Iwata T and Tsuge T 2016 Acs Sustainable Chem. Eng. 4 acssuschemeng.6b01851
[35] Liccariello G, Ferraro R, Russo M, Strozozi F, Catara A F, Bella P and Catara V 2017 New Biotechnol. 37 39
[36] Muangwong A, Boontip T, Pachimsawat J and Naphathorn S C 2016 Microbial Cell Factories 15 55
[37] Naveen S V, Shan G Y, Ping I T K, Murali M R and Kamarul T 2015 Mater. Lett. 141 55
[38] Zhuang Q, Wang Q, Liang Q and Qi Q 2014 Metabolic Engineering 24 78
[39] Kumar V, Gupta S, Mishra N K, Singh R, Yadav S K S and Joshi K B 2017 Mater. Res. Express 4 035036
[40] Li H Z K, Zhang H, Zhang W W, Li K and Xu J 2016 RSC Adv. 6 55618
[41] Nagappayya S K and Gaikar V G 2010 Ind. Eng. Chem. Res. 49 6547
[42] Zhu C, Hui D, Chen Q, Xue J, Shen S and Xia Y 2017 Adv. Mater. 29 1703702
[43] Wang C, Sauvageau D and Elias A 2016 Acs Applied Materials & Interfaces 8 1128
[44] Ma L, Zhang H, Liu Q, Chen J, Zhang J and Chen G Q 2009 Bioresource Technology 100 4891
[45] Dai Z W, Zou X H and Chen G Q 2009 Biomaterials 30 3075
[46] Kwiecien M, Adamsz G and Kowalcuk M 2013 Biomacromolecules 14 1181
[47] Fang T, Decker E A and Goddard J M 2013 Journal of Agricultural & Food Chemistry 61 12397
[48] Mauclaire L, Brombacher E, Bünger J D and Zinn M 2010 Colloids & Surfaces B Biointerfaces 76 104
[49] Arima Y and Iwata H 2007 Biomaterials 28 3074
[50] Lee J H, Lee J W, Kang G and Lee H B 1997 Biomaterials 18 351
[51] Chan R T H, Russell R A, Helder M A, Lee T H, Holden P J and Foster L J R 2014 Biomacromolecules 15 339
[52] Chiulan I, Mihaela P D, Helder M A, Teodosescu M, Nicolae C A, Cășărică A, Tofan V and Sălăgeanu A 2016 Journal of Biomedical Materials Research Part A 104 2576
[53] Lu X Y, Cirasolo E, Stefenia R, Chen G Q, Zhang Y and Hirsch E 2011 Applied Microbiology & Biotechnology 89 1423
[54] Jeevitha D and Amarnath K 2013 Colloids & Surfaces B Biointerfaces 101 126