Comparative Characterization of Polyhydroxyalkanoates and Fabrication of 3D-Printed Composite Scaffolds for Tissue Engineering

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Abstract

Polyhydroxyalkanoates (PHAs) are bio-polyesters that have gained significant potential for medical applications due to their properties, including biocompatibility, non-toxicity, and biodegradability within the human body. This study investigated the chemical and mechanical properties of various PHA types: short-chain-length PHA (SCL-PHA), medium-chain-length PHA (MCL-PHA), and poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx). The results indicated that SCL-PHA has high mechanical strength but lacks flexibility, whereas MCL-PHA exhibits low crystallinity, resulting in high elasticity. PHBHHx, a copolymer, demonstrates a balance of both strength and flexibility. Recognizing the potential of PHBHHx, the researchers used 3D bioprinting to fabricate scaphoid bone scaffolds using pure PHBHHx and PHBHHx composites with hydroxyapatite (HA) and tricalcium phosphate (TCP). An analysis of compressive strength before fracture was conducted, using the human bone standard of 2.6 MPa as a benchmark. The compressive strengths of the PHBHHx and the PHBHHx+HA+TCP composites were 2.051 ± 0.36 MPa and 4.130 ± 0.42 MPa, respectively. These findings clearly demonstrate that the incorporation of HA and TCP significantly enhances the load-bearing capacity of the scaffolds, highlighting their strong potential as bone substitute materials in regenerative medicine. Additionally, multi-layered 3D square scaffolds were constructed using MCL-PHA to assess biocompatibility and cellular proliferation, demonstrating that cells grown on these scaffolds had favorable growth patterns.

Keywords: *polyhydroxyalkanoates, physical properties, material composites, hydroxyapatite, 3D printing, scaphoid bone, biocompatibility*

1. Introduction

Polyhydroxyalkanoate (PHA) is an outstanding biopolyester with the ability to be stored inside various microorganisms (Tanadchangsaeng & Roytrakul, 2020; Yootoum et al., 2023). While it has similar properties to petroleum-based plastics, PHA is uniquely biodegradable (Park et al., 2012) and offers a customizable chemical structure, ranging from homopolymers to copolymers (Panaksri & Tanadchangsaeng, 2023, 2025). Consequently, its physical properties, such as mechanical strength and thermal stability, can be customized for specific applications (Suvarnaphaet et al., 2019; Thanomboonchaoen et al., 2024). For these reasons, PHA has gained significant attention in the medical field due to its biocompatibility, biodegradability, and non-toxicity to humans (Panaksri & Tanadchangsaeng, 2021, 2025). These qualities make it highly suitable for use as scaffolds and tissue-engineered constructs in regenerative medicine (Panaksri et al., 2023; Tawonsawatruk et al., 2023).

PHA can be categorized by monomer composition into homopolymers and copolymers. A homopolymer consists of only one type of monomer; the most well-known example is 3-hydroxybutyrate, or P3HB, which is characterized by its stiffness and brittleness. In contrast, a copolymer is a combination of two or more monomers, which offers an advantage for further development. Since both chemical and mechanical properties depend on the monomer structure, combining various monomers can address the limitations of pure polymers. For instance, incorporating monomer 3-hydroxyhexanoate (3HHx) into P3HB improves the mechanical properties by decreasing the brittleness and increasing the flexibility. Furthermore, PHA can be classified based on the chain length of hydroxy fatty acid monomers. Short chain length, SCL-PHA, consists of 3 to 5 carbon atoms; its properties are typically stiff and brittle, with high melting temperatures (Ray &

Kalia, 2017). In contrast, medium chain length, MCL-PHA, consists of 6 to 14 carbon atoms. This higher number of atoms results in low crystallinity, causing the material to behave as an elastomer with a low melting point (Ray & Kalia, 2017). To investigate the properties of various chemical structures of PHA, which can affect mechanical properties, several polymers were tested for their chemical and mechanical characteristics. The polymers used in this study are SCL-PHA, PHBHHx, and MCL-PHA.

PHA had been a significant candidate for applications in the medical field, as it possesses distinguishing properties such as biocompatibility and biodegradability (Moradali & Rehm, 2020). The primary use of PHA is as a scaffold for tissue regeneration, including tendon, bone, cartilage, blood vessels, and skin (Panaksri & Tanadchangsaeng, 2021). These scaffolds are designed to promote proliferation, cell viability, and tissue regeneration (Gadgil et al., 2017). The scaphoid bone, located in the human wrist, plays a critical role in connecting the carpal bones, facilitating complex movements, and distributing force across the wrist. For this reason, the scaphoid is the most commonly injured bone in the wrist. Among the polymers evaluated in this study, PHBHHx was selected for further development as a bone scaffold to address these clinical needs.

By incorporating a composite of hydroxyapatite (HA) and tricalcium phosphate (TCP) along with 3D bioprinting technology, scaffolds were fabricated (Jindapon et al., 2023; Panaksri et al., 2025). Both hydroxyapatite (HA) and tricalcium phosphate (TCP) are chemical compounds of calcium phosphate that are significant in medical and dental applications. They have chemical components similar to those of human bone and teeth. Continuously, they increased the strength of the scaffold and promoted the cell growth (Panaksri et al., 2025). The models of scaffolds were designed with various material ratios and shaped into a human scaphoid bone to perform compressive strength tests until failure (Kuncharin et al., 2019). Finally, MCL-PHA was molded into a multi-layer square by a 3D bioprinter to study cell viability and proliferation.

2. Objectives

This study was conducted to synthesis and physical properties testing of PHA and PHA/HA/TCP composites fabricated via 3D bioprinting for enhancing the properties of scaffold and cell biocompatibility studies of the PHA polymer.

3. Materials and Methods

Pseudomonas putida ATCC 47054 from a laboratory was used to synthesize MCL-PHA based on the previous study by Panaksri and Tanadchangsaeng (2021) through a fermentation process with glycerol and octanoate as co-substrates.

3.1 Chemicals and Reagents

The chemicals and reagents used in this study included *Pseudomonas putida* ATCC 47054, sodium peroxide (Na_2O_2), distilled water, nutrient broth, sodium dihydrogen phosphate (NaH_2PO_4), magnesium sulfate ($MgSO_4$), ammonium sulfate $(NH_4)_2SO_4$, dipotassium phosphate (K_2HPO_4), sodium octanoate ($CH_3(CH_2)_6COONa$), glycerol ($C_3H_8O_3$), trace element solution, chloroform ($CHCl_3$) and methanol (CH_3OH).

3.2 MCL-PHA Synthesis

Polyesters can be synthesized by various bacterial species; for instance, *Pseudomonas putida* ATCC 47054 is utilized to produce MCL-PHA under specific cultivation conditions. To facilitate PHA biosynthesis, the bacteria must be provided with sufficient carbon sources, such as glucose or fatty acids, while the nitrogen source is deliberately limited. This nutritional stress induces the microorganisms to accumulate the carbon source intracellularly in the form of PHA granules (Panaksri et al., 2024).

The cultivation process began with the activation of bacterial culture from -80°C storage. A bacterial sample of 1 mL was inoculated into a test tube containing 10 mL of nutrient broth and incubated at 30°C with agitation at 150 rpm for 24 hours. Subsequently, the culture was subcultured into 50 mL and 250 mL flasks containing mineral salt medium (MSM) composed of NaH_2PO_4 (1.2 g/L), $MgSO_4$ (0.5 g/L), $(NH_4)_2SO_4$ (2 g/L), and K_2HPO_4 (7.34 g/L). The medium was supplemented with glycerol 50% (v/v) to a final concentration

of 20 g/L, sodium octanoate ($CH_3(CH_2)_6COONa$) at 1 g/L from a 1% stock solution, and trace element solution. The cultures were then incubated at 30°C with shaking at 150 rpm for 48 hours.

For laboratory scale, a 5-L bioreactor was prepared with 2 L of mineral salt medium and 20 g/L of glycerol 50% (v/v) as the initial substrate. The bioreactor was equipped with probes for real-time monitoring of pH, temperature, dissolved oxygen (DO), and foam formation. The temperature was maintained at 30°C, while the impeller speed was adjusted between 200 and 800 rpm to maintain the pH at approximately 6.8. During the first 36 hours of cultivation, ammonium hydroxide was employed for pH control; thereafter, the base was switched to sodium hydroxide. After 24 hours of fermentation, a 1:1 (v/v) mixture of glycerol and sodium octanoate was fed continuously into the bioreactor at a rate of 0.1 to 0.2 mL/minute using an automated feeding system. Following 60 hours of total cultivation time, the culture was harvested by centrifugation at 6,000 rpm for 20 minutes, and the resulting biomass pellet was stored at -80°C for subsequent analysis.

3.3 MCL-PHA Purification

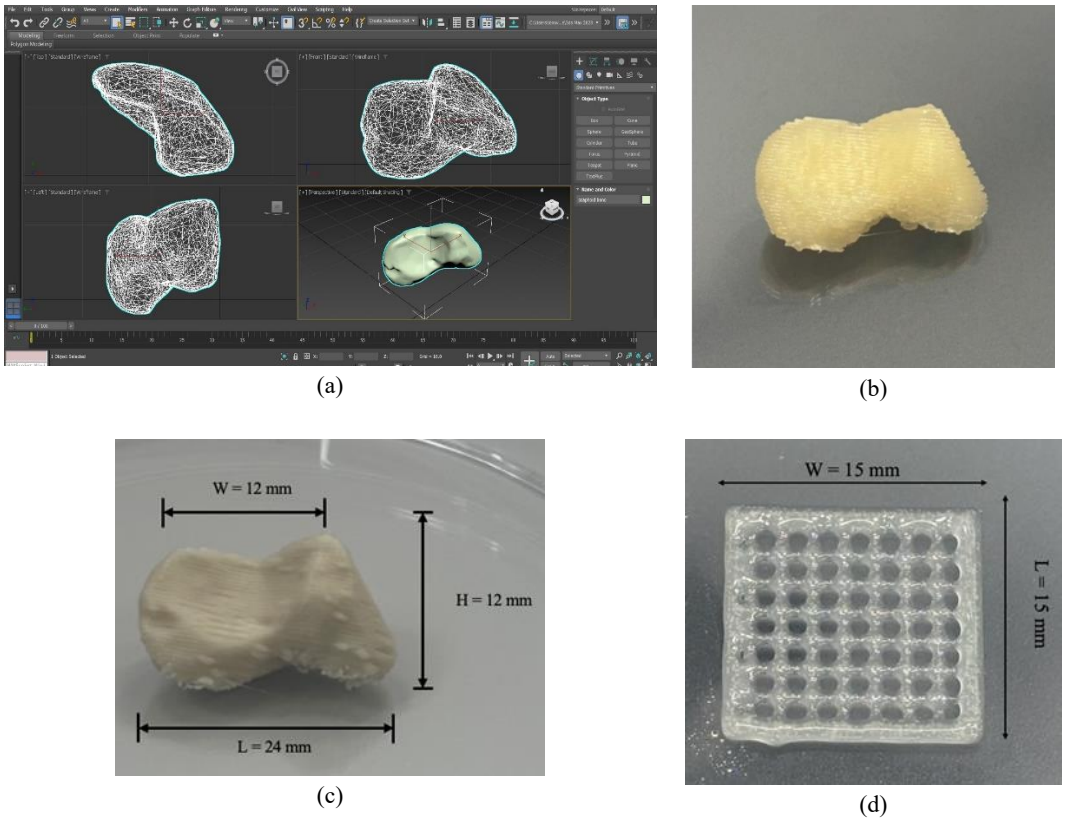
The preserved biomass pellet was freeze-dried for three days to ensure it was completely dry. Then, the dry biomass was ground into powder and dissolved in chloroform at a ratio of 1 gram of dry biomass per 100 ml of solvent. The mixture was stirred using a heated magnetic stirrer set to 50°C for 24 hours. Afterward, the solution was filtered through No. 1 filter paper and allowed to drop into chilled methanol. The volume of methanol used was 2 to 4 times the volume of the chloroform. During this process, the methanol was stirred continuously to facilitate the precipitation of MCL-PHA, which appeared as a white substance. This was the MCL-PHA produced during the fermentation process.

3.4 3D Bioprinting Scaffold

In cooperation with modern technologies, 3D bioprinting using a Dr. INVIVO 4D bioprinter (ROKIT Healthcare, Seoul, South Korea) was selected for this study. However, working with 3D bioprinting is quite challenging, as the parameters must be carefully adjusted. To begin with, the two models tested under this study were created using the 3Ds Max software, shown in Figure 1(a).

For the scaphoid model, the printing materials were pure PHBHHx and PHBHHx + HA + TCP composite with an 80:4:16 ratio (Panaksri et al., 2025), respectively. The melting temperature for these materials ranged from 140 to 160°C, while the bed temperature was set at 60°C, with the printing pressure maintained between 300 and 400 kilopascals. Details of the model were shown in Figure 1(b,c).

In contrast, the parameters for MCL-PHA differed significantly from those of PHBHHx. The melting temperature was lower, at approximately 60 to 70°C, the bed temperature was 0°C, and the pressure was set between 400 and 500 kilopascals. Infill density of MCL-PHA scaffold was 15% of size 15 x 15 mm. Porosity was 2 x 2 mm. The model fabricated for MCL-PHA was a multi-layered square featuring an internal grid structure as shown in Figure 1(d).



(a) (b) (c) (d)

Figure 1 (a) Model of scaphoid bone. (b) PHBHHx scaphoid bone. (c) PHBHHx + HA + TCP scaphoid bone and (d) Model of MCL-PHA.

3.5 Sterilization and Preparation of MCL-PHA for Testing on L929 Cells

The MCL-PHA scaffolds were sterilized by soaking in 70% ethanol for 15 minutes, rinsing three times with DPBS, drying in laminar airflow, and being exposed to 365 nm ultraviolet (UV) light for 40 minutes.

3.6 Chemical properties

3.6.1 Nuclear Magnetic Resonance or NMR

Nuclear Magnetic Resonance helps in identifying the molecular arrangement, chemical bonding, and microstructural features of PHA, which is crucial for understanding how these affect mechanical properties and degradation behavior when 25 mg of PHA is dissolved in 1 mL of deuterochloroform ($CDCl_3$). Deuterochloroform is a deuterated solvent commonly used in NMR because it provides minimal background interference and allows for clear detection of the polymer's spectra. The sample was subjected to ^{1}H NMR and ^{13}C NMR to analyze the hydrogen and carbon atoms in PHA. The duration of the experiment ranged from 30 minutes to one hour.

3.6.2 X-Ray Diffractometer or XRD

X-Ray diffractometer data can be used to calculate the crystallinity index, which indicates the proportion of crystalline material in the polymer compared to the amorphous phase. Higher crystallinity typically results in stiffer, stronger materials, while lower crystallinity may impart greater flexibility and faster biodegradability. The sample was cut into 3×3 cm^2. The XRD scan was performed over a 2θ range of 4° to 60°, where 2θ was the angle between the incident X-rays and the detector. The scan rate was set to 2°/minute, which provides a balance between data resolution and the time required for analysis.

3.6.3 Differential Scanning Calorimetry or DSC

The thermal analysis technique was used to measure how a polymer, like PHA, responds to changes in temperature. During the first heating step, the sample was heated from 25°C to 200°C. This range covers the melting point of PHA, allowing the identification of both the glass transition temperature (T_g), where the polymer changes from a glassy to a rubbery state, and the melting point (T_m), where the crystalline regions of the polymer melt. Then, the sample entered the cooling step when it was cooled from 200°C to -50°C. This cooling phase captures the crystallization temperature (T_c), where the polymer reorganizes into a crystalline structure upon cooling. Finally, the sample entered the second heating step when it was heated again from -50°C to 200°C. This step verifies the thermal properties recorded in the first heating phase and ensures that any thermal events (such as melting) were consistently observed.

3.7 Mechanical Properties Testing

3.7.1 Tensile Testing

To begin, PHA films with a thickness of 0.25 mm were cast and cut into rectangular strips measuring 5 × 50 mm. Sandpaper was attached to both ends of each strip to prevent slipping and to create a dumbbell-shaped testing area. After entering the sample parameters into the software, the specimen was secured in the machine's grips. The testing speed was then set to 100 mm per minute.

3.7.2 Compression Testing

The model used to test compression was the scaphoid bone model. The parameters of the model were measured and filled into the program. The testing speed was then set to 10 mm per minute.

3.8 Biocompatibility Testing

3.8.1 Cell Viability

Cell viability was analyzed to study cell behavior on the surface of the MCL-PHA scaffolds. The cell line used in this study was the L929 cell line (L929 mouse fibroblast cells, CLS Cell Lines Service GmbH, Germany) from XL Biotec Co., Ltd. (Bangkok, Thailand). To prepare the scaffolds, the fibroblast cells were first sterilized by soaking in 70% ethanol for 5 minutes, followed by rinsing with PBS three times. The scaffolds were then dried in a biosafety cabinet and UV sterilized on each side for 40 minutes.

For the cell viability assay, the L929 cells were seeded into wells containing the 3D scaffolds and supplemented culture media. The plates were then placed in a 5% CO_2 incubator at 37°C for 24 hours. After the incubation period, cell viability was assessed by staining with Trypan Blue. Finally, an inverted microscope was used to observe the cells and perform a cell count.

3.8.2 Cell Proliferation

Cell proliferation was analyzed to study the cell growth when seeding to MCL-PHA scaffolds. To prepare the scaffolds, they were first sterilized by soaking in 70% ethanol for 5 minutes, followed by rinsing with PBS three times. The scaffolds were then dried in a biosafety cabinet and UV sterilized on each side for 40 minutes.

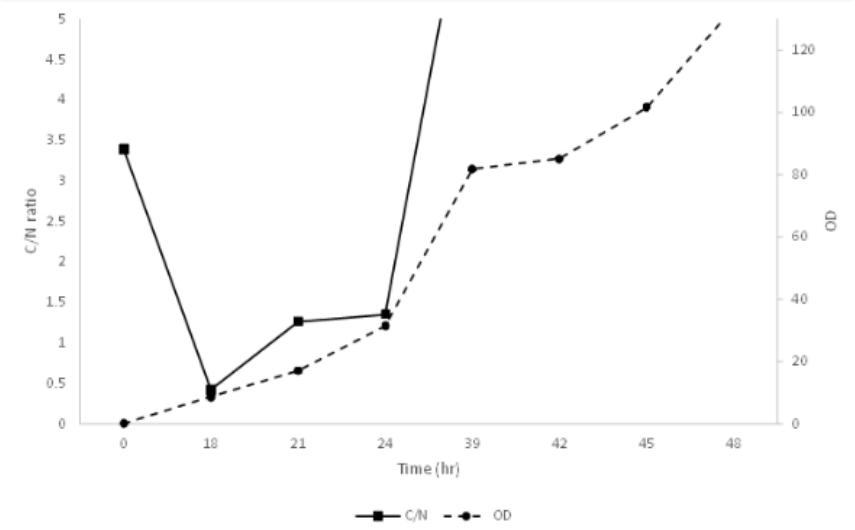
For the cell proliferation assay, the L929 cells with an amount of 3 x 10^4 cell were seeded into 6-well and supplemented with culture media. The plates were then placed in a 5% CO_2 incubator at 37°C for 24 hours. After the incubation period, cell viability was assessed by staining with crystal violet. Finally, an inverted microscope was used to observe the cell division.

4. Results and Discussion

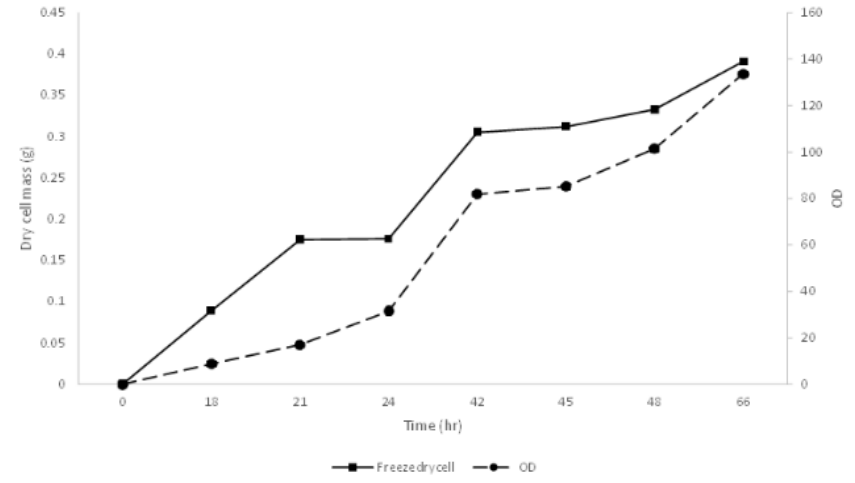
4.1 Laboratory Scale Synthesis of MCL-PHA

The MCL-PHA used in this study was synthesized in a 5-L bioreactor using glycerol and sodium octanoate as carbon sources. The resulting polymer consisted of 3HHx, 3HO, and 3HD monomers, following a production method adapted from previous research conducted by Panaksri and Tanachangsaeng (2021).

Pseudomonas putida ATCC 47054 was cultivated for 66 hours, revealing clear correlations between cultivation time, optical density (OD), and nutrient feeding profiles, as illustrated in Figure 2(b). The steady increase in dry cell weight (DCW) and the rise in OD between 18 and 39 hours confirm bacterial growth and MCL-PHA synthesis, which align with the carbon-to-nitrogen (C/N) ratios fed into the reactor (Figure 2(a)). Furthermore, a significant increase in OD following nitrogen limitation indicates enhanced polymer accumulation. These results demonstrate the capacity of *Pseudomonas putida* ATCC 47054 to utilize co-substrates for polymer production. While OD reflects the trend of bacterial proliferation, its increase specifically corresponds to polymer accumulation behavior as the C/N ratio rises—meaning nitrogen is restricted while carbon continues to be supplied for synthesis. The consistent relationship between OD and harvested DCW confirms that cell mass increased over time. The resulting dry cells were subsequently extracted and utilized for this study.



(a)



(b)

Figure 2 The relationship of time and growth of bacteria. (a) Relationship between time, OD, and supplement medium. (b) Relationship between dry biomass, time, and OD.

4.3. Chemical Properties

4.3.1 Nuclear Magnetic Resonance

To elucidate the influence of chemical structure on the material's properties, the PHBHHx copolymer was characterized using NMR spectroscopy. The ^{13}C NMR analysis identified the presence of two distinct monomers: 3-hydroxybutyrate (HB) and 3-hydroxyhexanoate (HHx). Quantitative analysis via ^{1}H NMR showed the molar composition to be 90.3% HB and 9.7% HHx.

For comparative analysis, the chemical composition of MCL-PHA was referenced from the previous study conducted by Panaksri and Tanachangsaeng (2021). According to the reported data, this polymer comprises three monomers: 3-hydroxyhexanoate (HHx), 3-hydroxyoctanoate (HO), and 3-hydroxydecanoate (HD), with a molar ratio of 3.3% HHx, 32.2% HO, and 64.5% HD.

The chemical structural analysis via NMR revealed significant differences between the two copolymers, which directly influence their thermal and mechanical properties. For PHBHHx, the high proportion (90.3%) of short-chain length monomers, specifically 3-hydroxybutyrate (HB, 4 carbons), serves as the primary backbone that promotes polymer chain alignment and high crystallinity, thereby imparting substantial mechanical strength to the material. Meanwhile, the incorporation of a minor fraction (9.7%) of medium-chain length monomers, such as 3-hydroxyhexanoate (HHx, 6 carbons), introduces steric hindrance that slightly disrupts structural regularity. This modification effectively mitigates the brittleness typically associated with pure PHB homopolymers, resulting in PHBHHx exhibiting a balanced profile of stiffness and flexibility, making it highly suitable for fabricating load-bearing bone scaffolds.

In contrast, the reference data for MCL-PHA indicate a composition dominated by monomers with higher carbon counts (6–10 carbons), particularly 3-hydroxydecanoate (HD), which possesses longer and bulkier side chains. This structural characteristic hinders the dense packing of polymer chains, leading to low crystallinity and elastomeric behavior. This finding aligns with the thermal and mechanical test results that are to be discussed subsequently.

4.3.2 Differential Scanning Calorimetry or DSC

The thermal properties of the synthesized PHBHHx were analyzed using DSC. The results revealed two distinct melting temperatures (T_m) at 114°C and 130.1°C, with a glass transition temperature (T_g) of 2.4°C. The enthalpy of melting (ΔH_m), representing the energy required for the phase transition, was calculated to be 32.2 J/g

For comparative purposes, the reference data for MCL-PHA indicate significantly lower thermal values, with a melting temperature of 52.21°C and a glass transition temperature of -49.35°C. The melting enthalpy for MCL-PHA was reported at 22.75 J/g. These thermal properties are summarized in Table 1

4.3.3 X-Ray Diffractometer or XRD

The crystalline structure of the polymers was evaluated using X-Ray Diffraction (XRD). The degree of crystallinity (X_c) was calculated based on the area under the crystalline peaks relative to the total area of the diffractogram. For PHBHHx, the analysis showed a degree of crystallinity of 40%, indicating a semi-crystalline structure. In contrast, the comparative data for MCL-PHA exhibited a much lower crystallinity of 15.7%, corresponding to a high amorphous content of 84.3%. The comparison of chemical, thermal, and crystalline properties between PHBHHx and MCL-PHA is presented in Table 1.

Table 1 Physical properties of PHBHHx and MCL-PHA.

	Ratio of monomer			Thermal properties			Crystallinity
	HB/HO	**HHx**	**HD**	**T_m (°C)**	**T_g (°C)**	**ΔHm (J/g)**	**X_c (%)**
PHBHHx	90.3 (HB)	9.7	-	114, 130.1	2.4	32.2	40
MCL-PHA	32.2 (HB)	3.3	64.5	52.21	-49.35	22.75	15.7

4.4. Mechanical Properties Testing

4.4.1 Tensile Testing

The PHA materials used in this study were SCL-PHA, PHBHHx, and MCL-PHA (Panaksri & Tanadchangsaeng, 2021). Regarding Young's modulus, SCL-PHA exhibited the highest value, followed by PHBHHx and MCL-PHA, with results of 145.29 ± 2.09 MPa, 112.48 ± 3.12 MPa, and 5.75 ± 1.10 MPa, respectively. In terms of ultimate tensile strength, SCL-PHA again showed the highest result at 12.20 ± 0.33 MPa, followed by MCL-PHA at 4.93 ± 0.92 MPa, and PHBHHx at 4.88 ± 0.15 MPa. However, for elongation at break, MCL-PHA reached the highest percentage at 278 ± 0.55%, significantly higher than PHBHHx (17.5 ± 0.04%) and SCL-PHA (12.22 ± 0.03%). The result summaries are present in Table 2.

Table 2 Mechanical properties of different PHAs via tensile test.

	Young's modulus (MPa)	**Ultimate strength (MPa)**	**Elongation at break (%)**
SCL-PHA	145.29 ± 2.09	12.20 ± 0.33	12.22 ± 0.03
PHBHHx	112.48 ± 3.12	4.88 ± 0.15	17.5 ± 0.04
MCL-PHA	5.75 ± 1.10	4.93 ± 0.92	278 ± 0.55

The chemical analysis of PHBHHx indicated that this copolymer consists of both SCL-PHA and MCL-PHA monomers. The crystalline region was approximately 40%, which directly affects the thermal properties of the polymer; generally, higher crystallinity leads to higher melting and glass transition temperatures. Additionally, crystallinity significantly influenced mechanical performance. These findings clearly demonstrate that the differences in chemical structure between PHBHHx and MCL-PHA impact their tensile results. Specifically, since MCL-PHA has a lower crystalline region, it exhibits a higher elongation at break and a lower melting point compared to PHBHHx.

4.4.2 Compression Testing

The compression testing results clearly demonstrate that the incorporation of Hydroxyapatite (HA) and Tricalcium Phosphate (TCP) into the PHBHHx matrix significantly enhanced the compressive strength, increasing it from 2.051 ± 0.36 MPa in the pure PHBHHx to 4.130 ± 0.42 MPa in the composite material. This enhancement is attributed to the inherent stiffness and high compressive resistance of the ceramic particles (HA/TCP). These particles function as a reinforcement phase within the polymer matrix, effectively distributing applied loads and retarding structural deformation under stress.

In comparison to the physiological compressive strength of the human scaphoid bone, reported at approximately 2.6 MPa by Faragó & Kiss (2020), the PHBHHx/HA/TCP composite exhibited a strength approximately 1.5 times higher than this benchmark. This finding indicates its suitability and safety for use as a bone substitute in load-bearing applications. Conversely, the pure PHBHHx scaffold fell slightly below this physiological standard, suggesting that clinical application might require supplementary external fixation. However, the strength of the composite scaffold (4.13 MPa), while exceeding that of natural bone, remains within an acceptable range and is not excessive enough to induce severe stress shielding effects on adjacent bone tissue.

Beyond ultimate strength, the stress-strain curves reveal that the composite material (HA-TCP) can sustain higher strain levels before failure compared to the pure PHBHHx. This behavior demonstrates that the synergistic combination of the flexible PHBHHx matrix and the rigid HA/TCP fillers within the 3D-printed architecture enhances the material's toughness and energy absorption capacity. These properties are critical for implants subjected to the dynamic loading conditions characteristic of the human wrist. However, in the real situation, the force and the direction that could break the bone can be in various ways. Therefore, in the future, it would be highly recommendedto test the compression in different directions.

4.5 Biocompatibility Testing

4.5.1 Cell Viability

PHA-based scaffolds have shown no apparent cytotoxicity and outstanding biocompatibility with a variety of cell types, including but not limited to bone, cartilage, neuron, heart, and pancreatic (Gregory et

al., 2022). In this study, L929 cells were used exclusively to assess cell adhesion behavior on the scaffold surface. L929 cell viability was used to assess cell culture with MCL-PHA scaffolds in growth media for 24 hours. After the incubation period, the cells were stained with Trypan Blue and observed under an inverted microscope at 4X magnification, as shown in Figure 3, to assess viability and perform cell counts. The results revealed a high survival rate, indicating that the cells thrived when cultured with the scaffolds.

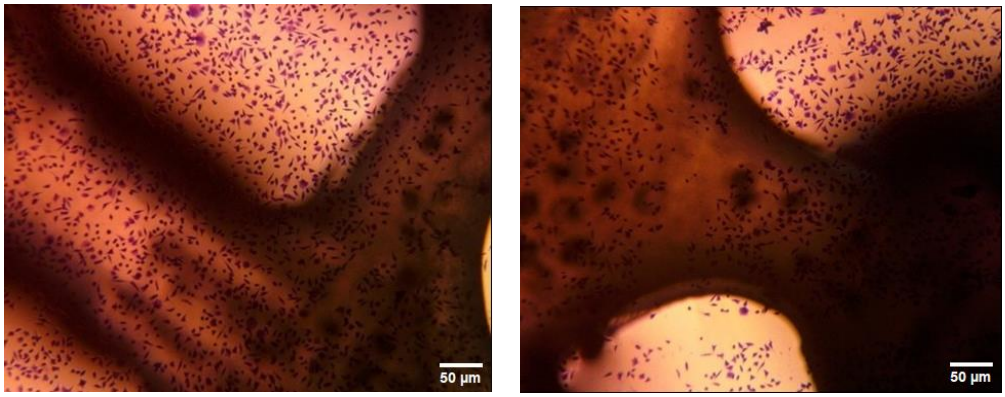
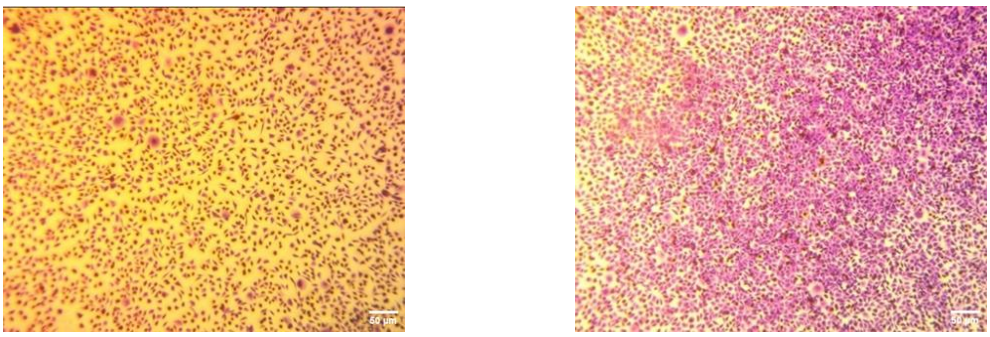


Figure 3 The images of cell viability of L929 cells cultured with MCL-PHA under an inverted optical microscope of 4X magnification. Scale bar: 50 µm.

Microscopic observation further confirmed that the fibroblasts maintained their characteristic spindle-shaped morphology, adhering well to the scaffold surface without signs of rounding or detachment, which are typical indicators of cytotoxicity. This preservation of cellular morphology suggests that the MCL-PHA surface provides a favorable physicochemical environment for initial cell attachment, effectively mitigating potential adverse effects from trace impurities.

4.5.2 Cell Proliferation

In the cell proliferation assay, L929 cells were cultured with MCL-PHA scaffolds in growth media for 24 hours. The cells were then stained with crystal violet solution and observed under an inverted microscope at 4X magnification, as shown in Figure 4. The results demonstrated a high survival rate for cells cultured with scaffolds. Notably, the cell density in the MCL-PHA group was found to be comparable to that of the control group, further confirming the material's biocompatibility. However, the process used in this study has potential limitations. Firstly, the cell culture facilities were inadequate due to unstable CO_2 levels that should be at 5%. The other limitation was time constraints which resulted in abnormal cell morphology.



(a) (b)

Figure 4 The staining image results of cell proliferation of L929 cells cultured with MCL-PHA. (a) L929 cells (b) L929 cells cultured with MCL-PHA under an inverted optical microscope of 4X magnification. Scale bar: 50 µm.

The intense crystal violet staining observed in the MCL-PHA group reflects a substantial accumulation of biomass, indicating active cell division rather than mere stasis. The ability of L929 cells to proliferate to near-confluence on the material surface demonstrates that the chemical composition and topography of the MCL-PHA scaffold do not induce apoptotic signaling or growth inhibition, thereby fulfilling a fundamental requirement for tissue engineering scaffolds intended to support long-term tissue regeneration.

5. Conclusion

The laboratory-scale synthesis of medium-chain-length polyhydroxyalkanoate (MCL-PHA) was successfully achieved using *Pseudomonas putida* ATCC 47054 with glycerol and sodium octanoate as co-substrates, where monitoring of optical density (OD) confirmed that polymer accumulation occurred effectively as bacteria entered the stress phase. Chemical and physical characterization via NMR, DSC, and XRD illustrated the distinct behaviors of the copolymers; PHBHHx, composed of SCL and MCL monomers, exhibited high crystallinity and stiffness, whereas MCL-PHA, comprising three distinct MCL monomers with longer chain lengths, demonstrated lower melting temperatures and elastomeric properties. Leveraging these high-strength characteristics, PHBHHx was utilized to fabricate 3D-bioprinted scaphoid bone models, where the incorporation of hydroxyapatite (HA) and tricalcium phosphate (TCP) composites yielded satisfactory compressive strength results suitable for load-bearing applications. Nevertheless, future studies must address multiaxial loading to ensure comprehensive structural integrity. Concurrently, the biocompatibility of the elastomeric MCL-PHA was validated through multi-layered 3D scaffolds, where cell viability and proliferation assays demonstrated a positive growth trend, confirming that the material provides a supportive, non-toxic environment essential for tissue regeneration.

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