Cell Compatibility of Medium-chain-length Polyhydroxyalkanoate as A Biomaterial: Viability, Attachment, and Proliferation

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Abstract

Medium-chain-length polyhydroxyalkanoate (MCL-PHA) is a polymer derived from bacteria that has the ability to biodegrade and biocompatibility and has received significant attention for its potential use as a medical material. MCL-PHA possesses material properties of elastomers, making it a suitable material for engineering highly flexible tissue scaffolds. Confirming compatibility with cells is crucial for the application of MCL-PHA produced, as various types of MCL-PHA produced under different conditions cannot confirm the biocompatibility of all MCL-PHA produced. This study tested the compatibility properties of MCL-PHA with mesenchymal stem cells (MSCs) to examine cell viability, cell attachment, and cell proliferation. The results showed that MSCs fed with a cell culture medium containing MCL-PHA had 100% cell viability, which was not significantly different from the control group. The cell attachment tests demonstrated the extent of attachment of MSCs on the MCL-PHA film, while the cell proliferation tests showed a significant difference ($p < 0.001$) in the number of MSCs co-cultured with cell culture medium containing MCL-PHA at 24 h, 48 h, and 72 h compared to the control group, indicating that the rate of cell growth did not differ from the control group. Therefore, it can be concluded that MCL-PHA produced is compatible with cells.

Keywords**:** *Medium-chain-length polyhydroxyalkanoate, Biocompatibility, Cell viability and proliferation, Biomedical materials, Microbial polyesters*

1. Introduction

Medium-chain-length polyhydroxyalkanoate (MCL-PHA) is a type of biopolyesters produced by microorganisms (Silva et al., 2021). These biopolymers comprise hydroxyalkanoic acid monomers and have a medium chain length, typically ranging between 6 and 14 carbon atoms (Albuquerque & Malafaia, 2018). Due to their biodegradability and similar properties to conventional plastics, MCL-PHA has been gaining attention as a sustainable alternative to traditional plastics (Khatami et al., 2021). Researchers have recently investigated the use of MCL-PHAs in various applications, including biomedical implants and tissue engineering (Gregory et al., 2022). In the previous study, MCL-PHA was produced from *Pseudomonas putida* ATCC47054 bacteria as a material for creating 3D-printing scaffolds. The resulting MCL-PHA is a terpolyester with elastomeric properties. The evaluation of MCL-PHA as a biomaterial ink showed the potential to create highly flexible scaffolds using a 3D bioprinter (Panaksri & Tanadchangsaeng, 2021). MCL-PHA demonstrated potential as an engineering material for tissue engineering in terms of its outstanding shaping and mechanical properties. A critical aspect of using these biopolymers in these applications is ensuring they are compatible with cells and tissues.

Cell compatibility is a critical factor in determining the biocompatibility of a biomaterial. It refers to the ability of cells to grow and function properly when in contact with the material. Different microorganisms using different compounds can produce different types of MCL-PHAs, so their cell compatibility may vary. The *Pseudomonas* bacterial strain employed to produce MCL-PHA is a gram-negative bacterium that contains endotoxin as an internal component within its cells. Endotoxin is a toxic

substance that can impact the body immune system and is released when bacterial cells are destroyed. Therefore, testing the compatibility of the MCL-PHA cells produced by the bacteria, as mentioned earlier, is essential (Chen & Zhang, 2018). It is necessary to confirm and evaluate the cell compatibility of specific MCL-PHA types for specific biomedical applications (Singh et al., 2019).

In this study, we review the current state of research on evaluating the cell compatibility of MCL-PHAs, focusing on studies assessing cell attachment, proliferation, and viability. Specifically, we discussed how the produced MCL-PHAs affect cell behavior, including adhesion, proliferation, and viability. Cell compatibility testing was performed by Human mesenchymal stem cells (MSCs). By exploring the current understanding of MCL-PHA cell compatibility, we aim to provide a comprehensive overview of the potential of these biopolymers in the field of biomedicine and to identify the challenges that must be overcome to realize their potential as sustainable biomaterials fully.

2. Objectives

The three main objectives of this study were to test the cell viability of MCL-PHA, to assess the ability of cells to adhere to MCL-PHA, and to determine whether MCL-PHA inhibits cell proliferation.

3. Materials and Methods

The MCL-PHA sample from a laboratory mutant of *Pseudomonas putida* ATCC47054 used in this study was produced from the previous study of Panaksri and Tanadchangsaeng, 2021 by fermentation process with glycerol and octanoate as co-substrates (Panaksri & Tanadchangsaeng, 2021).

3.1 Chemicals and reagents

Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), and Penicillin-streptomycin were purchased from Gibco (Thermo Fisher Scientific, USA). Dimethyl sulfoxide (DMSO) and Thiazolyl Blue: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich, Germany.

3.2 Cell culture condition

Bone marrow-derived mesenchymal stem cells (BM-MSCs) were isolated from subjects during operation, as described in our previous research (Tawonsawatruk et al., 2021). The procedure was approved by the ethical committee of the Faculty of Medicine, Ramathibodi Hospital, Mahidol University (MURA2017/603) (Phetfong et al., 2021). The MSCs were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco), supplemented with 10% fetal bovine serum (FBS, Gibco) and penicillin (100 U/ml) / streptomycin (100 µg/ml) (Gibco). Cells were maintained at 37 °C in an atmosphere of humidified air with 5% CO_2.

3.3 Sterilization and preparation of MCL-PHA for testing on MSCs

The MCL-PHA films were sterilized by soaking in 70% ethanol for 5 min, rinsing three times with PBS, drying in laminar airflow, and being exposed under ultraviolet (UV) light for 40 min. MCL-PHAs were cut off a small piece, weight, and added the culture medium with the final concentrations at 1, 10, and 100 mg/ml. MCL-PHAs were incubated in a CO_2 incubator for three days before the experiment to assess cell viability. For cell adhesion testing, the sterilization was done as above and cut into circles with a diameter of 0.8 cm for putting on 48 wells plate.

3.4 Cell viability

The determination of the MCL-PHA sample on cell viability was performed by MTT assay (Lewandowski et al., 2022). MSCs were suspended and seeded as 100 µl in 96-well plates at 5 x 103 cells/well density and incubated overnight. Cells were treated with varying concentrations of the MCL-PHA samples in a culture medium at 1, 10, and 100 mg/ml for 24 h. MTT solution was prepared by dissolving with PBS at 5 mg/ml for 100 µl in each well, followed by 100 µl of culture medium as a final concentration of 2.5 mg/ml, and then incubated for 1 h. DMSO was used for dissolving the formazan crystals formed, and the quantity of

the colored formazan derivative was determined by measuring absorbance at 570 nm with 620 nm as a reference filter. The experiment was performed in three replicates, and the percentage viability was calculated as % viability = [OD of treated cells/OD of control cells] ×100 (Zhang et al., 2014).

3.5 Cell attachment

The evaluation of cell attachment to the surface of the MCL-PHA sample was done by cell adhesion assay. MCL-PHA sample was sterilized and put on each well of 48 wells plate. MSCs were seeded at 3 x 104 cells/well density and incubated for 24 h. Cells were fixed with 100% ice-cold methanol for 20 min and washed 2 times with ice-cold PBS. Cells were stained with 0.5% crystal violet for 15 min. The dye stain was removed and rinsed with distilled water several times until the color was cut off, and let the MCL-PHA sample air dry for a few minutes. Cell adhesion on the MCL-PHA samples was done in three replicates and photographed under the inverted microscope with magnifications of 4X and 10X.

3.6 Cell proliferation

Sterile MCL-PHA polymer materials (Sterilization) by immersing them in 70% alcohol for 5 minutes, washing with PBS 3 times, drying in a biosafety cabinet, exposing both sides to UV light for 40 minutes, and then cutting into small pieces. Then weighed 1 gram and immersed in cell culture without FBS for 3 days before the cell proliferation test. Prepare 1 x 10^3 cells/wells in 96 wells plates for testing at 24, 48, and 72 hours. MSC cells are mixed with 10% FBS cell culture, and the cell culture is immersed in MCL-PHA, in which the final concentration is 1, 10, and 100 mg/mL—then cured in the cabinet at 5% CO_2, 37 °C, within the specified time. After that, each image was taken and counted using trypsin to let the cell fall off the flask surface, then dye the cell with trypan blue and counted the cell with a machine. Hematocytometer was performed with three repetitions, calculated, and plotted using 2way ANOVA and Prism5 statistics, respectively.

4. Results and Discussion

4.1 Cell viability assessment of the MCL-PHA sample

MTT assay was examined to confirm the viable cells in the MCL-PHA sample's culture medium. MCL-PHAs were evaluated with varying concentrations to observe and compare the cytotoxic effect with untreated cells. The morphology of MSCs treated with the MCL-PHA sample and the untreated group was shown no difference between the groups (Figure 1). The percentage of cell viability was demonstrated with 100 percent viable cells for both MSCs treated and untreated cells. MCL-PHA has shown no statistically significant difference in the cytotoxic effect on MSCs (Figure 2).

The cell viability was calculated from the absorbance value of purple formazan crystals obtained from the solution. The cell viability of the control group was not different from the cells cultured with different concentrations of MCL-PHA solutions. The results indicate that the levels of mitochondrial dehydrogenases in all groups were similar before being converted to formazan crystals by MTT. The test suggests that MSCs use mitochondrial dehydrogenases in cellular respiration without being inhibited by MCL-PHA.

4.2 Evaluation of cell attachment on MCL-PHA sample

Cell attachment was monitored for evaluation of the MCL-PHA sample biocompatibility on MSCs. The result showed that the MCL-PHA sample has a suitable property for cell adhesion, as shown in Figure 1. The morphology of MSCs with or without the MCL-PHA sample showed no difference (Figure 3a). Cells were fixed, stained with 0.5% crystal violet, and photographed under an inverted microscope. The result has seen that many stained cells were detected at the magnification of 10X (Figure 3b). This result confirmed that the MCL-PHA sample has excellent biocompatibility to adhere to MSCs.

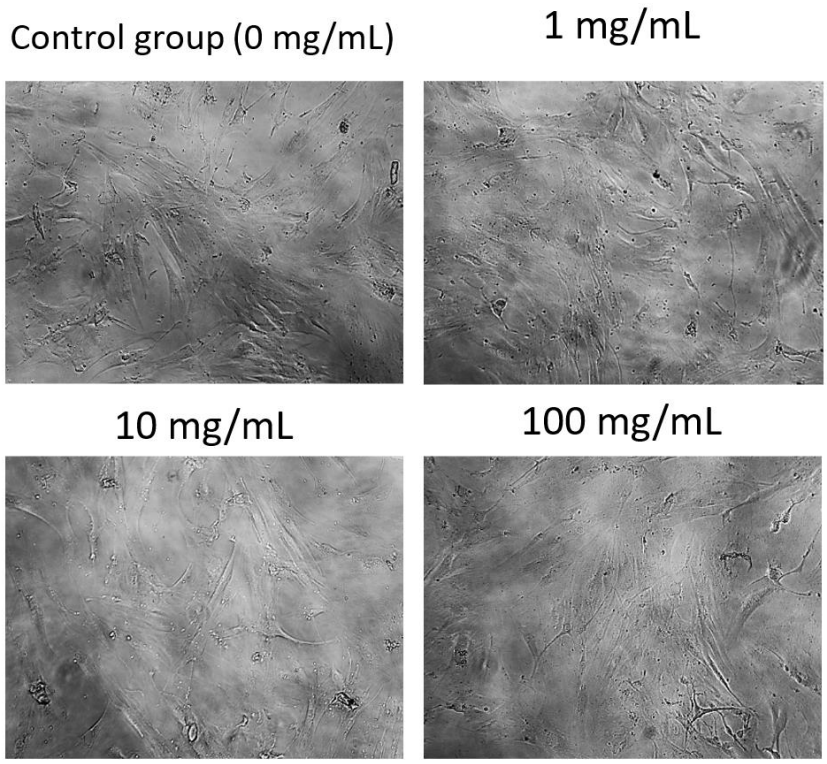

Figure 1 The morphology of MSCs treated with the MCL-PHA sample and the untreated group showed no difference between the groups

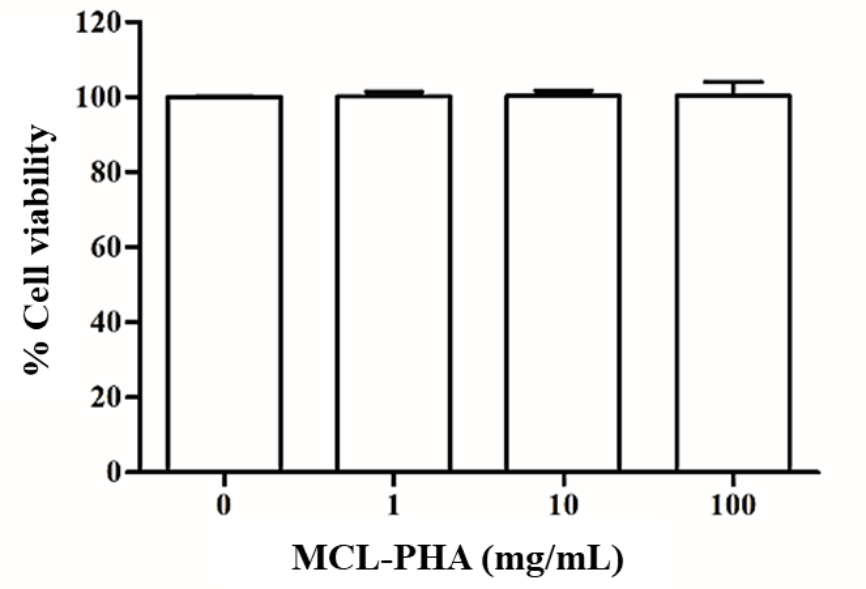

Figure 2 The cell viability percentage of MSCs cultured in MCL-PHA soaked in different concentrations compared with the control group

Figure 3a indicates that MCL-PHA cells can adhere to the surface of MCL-PHA films, similar to the surface of cell culture plates. The black line in the figure shows the edge of the MCL-PHA film that separates the cell culture area from the film area. The MCL-PHA film has a transparent characteristic that allows the observation of cells adhered to its surface through an inverted microscope, which is no different from cells adhered to the surface of cell culture plates. Figure 3b shows a clear view of MSCs on the MCL-PHA film that was stained with crystal violet. The staining results indicate that cells can proliferate and adhere to the surface of the MCL-PHA film.

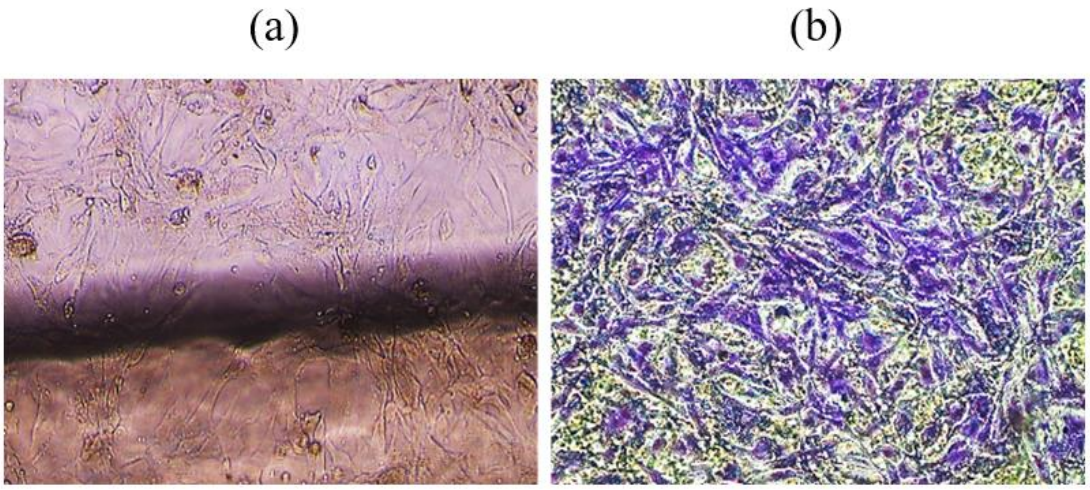

Figure 3 The morphology of MSCs on MCL-PHA sample at the magnification 10X: (a) without crystal violet stains and (b) crystal violet stains sample

4.3 Results of cell proliferation tested with MCL-PHA

MCL-PHA polymer materials were studied for effects on cell proliferation using high, medium, and low concentrations (1, 10, 100 mg/ml) and tested at 24, 48, and 72 hours (Figure 4). The results from cell counting and analysis by Two-Way ANOVA showed that the concentration variable did not result in a significant difference in cell numbers (Figure 5). When considering the time variable, a significant difference in cell numbers was found at a significant level of less than 0.001. From the above results, it can be concluded that MCL-PHAs do not affect inhibiting cell proliferation.

The cell proliferation assay results showed that MCL-PHA did not affect cell growth inhibition, even at higher concentrations. MSCs could normally proliferate, which correlated with the increased number of cells at 0 to 72 hours. The normal growth rate of cells dividing to increase their numbers confirmed that MCL-PHA is compatible with cells in-vitro.

The cell viability and proliferation results of MSCs proved the non-toxicity of MCL-PHA, which was consistent with the results of cell viability assays with MSCs previously studied in research (Naveen et al., 2015). Also, MCL-PHA was previously tested with cardiac progenitor cells (CPCs) and had good cell proliferation results (Constantinides et al., 2018). These data show that the material is compatible with other cells, indicating good cellular compatibility. MSCs have good adherence to the material, which is a property that tissue engineering requires (Zamri et al., 2021). However, more studies are needed to evaluate the long-term effects of MCL-PHAs on cells and tissues, such as in-vivo testing, as well as to optimize the processing and production of MCL-PHAs for specific applications.

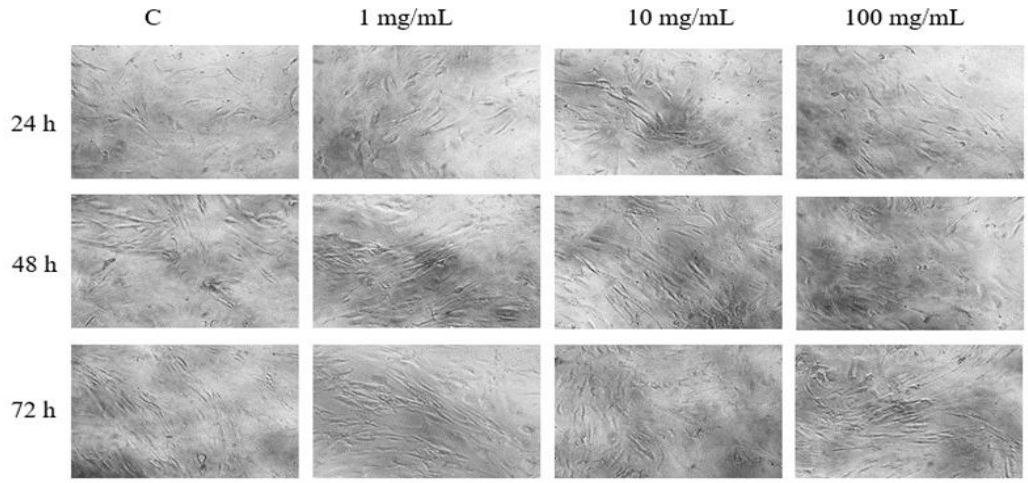

Figure 4 Cell proliferation of 24, 48, and 72 h characterized by cultured cells using cell culture media containing cells immersed in MCL-PHAs at various concentrations and times compared with the untreated MSCs of the control group

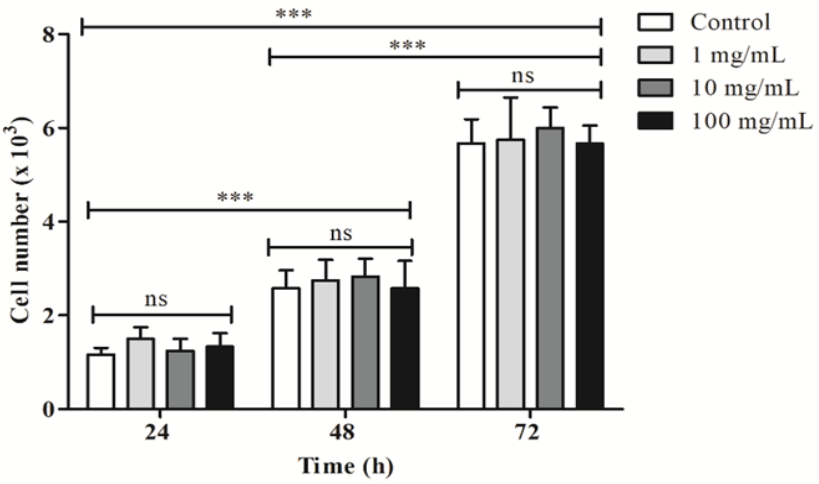

Figure 5 Cell counting results at various concentrations and times, with the concentration of MCL-PHA polymer material that does not affect the inhibition of cell proliferation. While the cells increase in time increasing respectively (n = 3, ns = Not Significant, *** = p <0.001)

5. Conclusion

This study demonstrated the excellent cell compatibility effect of medium-chain-length polyhydroxyalkanoate (MCL-PHA) as a biomaterial. The MTT assay confirmed that the MCL-PHA sample had no cytotoxic effect on mesenchymal stem cells (MSCs), and the morphology of MSCs treated with MCL-PHA and untreated cells were similar. The cell attachment assay showed that the MCL-PHA sample had excellent biocompatibility for cell adhesion, confirmed by the crystal violet staining results. Furthermore, the cell proliferation experiment revealed that MCL-PHA had no inhibitory effect on cell proliferation, indicating its potential as a suitable material for tissue engineering applications. The results of this study suggest that MCL-PHA is a promising biomaterial with great potential in tissue engineering. Further studies can focus on exploring the in vivo performance of MCL-PHA and investigating its potential as a scaffold material for tissue regeneration.

6. Acknowledgements

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