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Biocompatibility Assessment of Blue Light-Activated Methacrylated Hyaluronic Acid Hydrogel with L929 Fibroblast Cell Line

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Abstract

Objectives: To evaluate the biocompatibility of blue light-activated methacrylated hyaluronic acid (BL-MeHA) hydrogel using the L929 cell line.

Methods: Biocompatibility was assessed using three different assays. In the indirect cytotoxicity assay, L929 cells were cultured in conditioned media that had been exposed to BL-MeHA for 24 hours, followed by an MTT assay to evaluate cell viability. In the 2D culture assay, L929 cells were seeded on top of the BL-MeHA hydrogel, and cell viability was measured on days 1, 3, 5, and 12 using the resazurin assay. For the encapsulation culture assay, L929 cells were embedded within the BL-MeHA hydrogel, and viability was similarly assessed on days 1, 3, 5, and 12 using the resazurin assay. Additionally, L929 cell morphology was examined using scanning electron microscopy (SEM).

Results: The indirect cytotoxicity assay demonstrated that L929 cells remained viable when cultured with the BL-MeHA extract. In both the 2D and encapsulation culture assays, L929 cells initially exhibited slower growth compared to the control group but reached comparable levels by day 12. Notably, there was no significant difference in cell viability between BL-MeHA samples cured for 60 and 90 seconds.

Conclusions: The BL-MeHA hydrogel exhibited no cytotoxic effects on L929 cells, indicating good biocompatibility. These findings support its potential use as a scaffold for future applications in cell encapsulation or drug delivery for soft tissue engineering.

Keywords: cell viability, hyaluronic acid, hydrogel, L929 cell

Introduction

The goal of tissue engineering is to regenerate new tissue at the site of injury to restore its original function.[1,2] Tissue loss may involve either soft tissue or hard tissue, such as bone, with the latter being particularly challenging to replace.[3-6] Tissue engineering relies on three fundamental components: cells, scaffolds, and biologically active molecules. The effectiveness of a scaffold depends on various factors, including its surface and bulk properties, as well as its clinical practicality. An ideal biomaterial should possess several key characteristics, it must be biocompatible, degrade into non-toxic by products, and avoid triggering an immune response. Poor biocompatibility can lead to immune activation, inflammation, or even tumour formation.[6] Biodegradability refers to the chemical or enzymatic breakdown of a material within the body over time. Ideally, these materials should break down into harmless substances that can be safely eliminated from the body.[1]

A wide range of biological scaffold materials has been developed for use in both soft and hard tissue engineering. Among these, hydrogels are among the most widely utilized due to their many advantageous properties. Their hydrophilic nature allows them to absorb water and swell efficiently, making them particularly suitable for drug delivery and tissue engineering applications.[7-9] Hydrogels can be synthesized from a variety of precursors, including polyethylene glycol, alginate, polyvinyl alcohol, chitosan, heparin, and hyaluronic acid. Hyaluronic acid-based hydrogels, in particular, offer excellent biocompatibility due to their similarity to components of the extracellular matrix. They can be enzymatically degraded within the body, and their structure can be modified to enhance physical characteristics, making them highly adaptable for a wide range of biomedical applications.[10-12]

In 2018, Trakiattikul *et al.,*[13] developed a methacrylated hyaluronic acid (MeHA) hydrogel incorporating mannitol and bovine serum albumin. Although the hydrogel demonstrated favourable physical properties, its gelation time of approximately 30 minutes was too long to be clinically practical. Subsequent studies showed that this MeHA hydrogels crosslinked with dithiothreitol (DTT) were biocompatible with human alveolar bone cells and human gingival fibroblasts.[14,15] However, the prolonged gelation time of 15-30 minutes remained a limitation for future clinical use. To address this issue, Chaopanitcharoen *et al.,*[16] in 2023 incorporated lithium trimethyl benzoyl phosphinate (LAP) into MeHA to develop a blue light- activated MeHA (BL-MeHA) hydrogel system, enabling better control over gelation time. Their results demonstrated that 90 to 120 seconds of light exposure produced the hydrogels with desirable physical properties, including uniform pore size and effective swelling behaviour, highlighting their potential as scaffolds for clinical application.

The objective of this study was to evaluate the biocompatibility of BL-MeHA hydrogel using the L929 mouse fibroblast cell line, in accordance with the International Standards Organization (ISO 10993-5) guideline for biomedical materials testing. The findings are intended to support the development of effective biological scaffolds for tissue engineering applications.

Materials and Methods

BL-MeHA hydrogel preparation and blue light activation

The BL-MeHA hydrogel was synthesized based on the protocol described by Chaopanitcharoen *et al.,*[16] Briefly, 1% (w/v) of 47-kDa hyaluronic acid (HA) was dissolved in a potassium phosphate buffer (pH 8). Methacrylic anhydride (Me, MW 154.16 g/mol) was then added dropwise at a 1:10 molar ratio relative to HA. The reaction was conducted at 4°C for 24 hours under continuous stirring with a magnetic stirrer. The resulting MeHA solution was transferred into dialysis tubing and dialyzed to remove unreacted substances. After purification, the solution was collected, flash-frozen, and lyophilized (Labconco lyophilizer, Missouri, USA) for 3 days. Multiple batches of the gel were synthesized, and samples were sent for analysis of the degree of modification using proton nuclear magnetic resonance (^{1}H NMR) spectroscopy at the Scientific and Technological Research Equipment Center, Chulalongkorn University, Bangkok, Thailand. For the light curing unit, 3M™ Elipar™ S10 LED was used as the blue light source throughout this study.

Cell culture condition

L929 mouse fibroblast cells obtained from the American Type Culture Collection (Manassas, VA, USA) were maintained in Dulbecco's modified eagle's medium (DMEM: Ward Medic) containing 10% fetal bovine serum

(Gibco™) and 1% penicillin–streptomycin (Invitrogen) (10%FBS-DMEM). The culture was kept in 5% CO_2 37°C incubator.

Indirect cytotoxicity assay

The BL-MeHA hydrogel was cast in a plastic mold to form a cylindrical shape of 20 mm in diameter and 5 mm in thickness. A blue light curing time of 90 seconds was used. Following curing, the hydrogel was soaked in 20 mL of serum free Dulbecco's modified eagle medium (SF-DMEM) for 24 hours. The extract conditioned medium was collected and subsequently diluted to concentrations of 50%, 25% and 12.5% in SF-DMEM, with the original undiluted medium representing 100%.

L929 fibroblasts were plated at a density of 10,000 cells per well in a 96-well plate and allowed to attach overnight. The following day, the cells were treated with 100 µL of BL-MeHA hydrogel conditioned media at concentrations of 100%, 50%, 25% and 12.5%. Additionally, 10% dimethyl sulfoxide (DMSO) and 10% FBS-DMEM were used as control groups to represent toxic and non-toxic conditions, respectively. After 24 hours of incubation, cell viability was assessed using the MTT assay. Three separate experiments were performed in triplicate.

2D culture assay

A volume of 80 µL of BL-MeHA hydrogel was formed at the bottom of each well in a 96-well plate using blue light irradiation for either 60 or 90 seconds. Then, 100 µL of serum-free DMEM (SF-DMEM) was added to each well and incubated for 24 hours. After incubation, L929 fibroblasts were seeded onto the hydrogel surface at a density of 5,000 cells per well. The cell culture medium (10% FBS-DMEM) was refreshed every two days. Cell viability was assessed using the resazurin reduction assay on days 1, 3, 5, and 12 after cell seeding. The resazurin solution (PrestoBlue™, Invitrogen; Thermo Fisher Scientific) was prepared according to the manufacturer's instructions. The control group consisted of L929 cells cultured directly on the surface of a 96-well plate. All experiments were performed in three independent replicates.

Encapsulation culture assay

Following lyophilization and sterilization with 99.99% ethanol, the prepared hydrogel was immersed in 10% FBS-DMEM, and LAP was added at a final concentration of 15 mg/L. A total of 100 µL of the BL-MeHA hydrogel mixture was combined with 2×104 L929 cells suspended in 100 µL of 10% FBS-DMEM. The mixture was gently pipetted up and down to ensure a homogeneous gel–cell suspension, which was then seeded into a 96-well plate. Polymerization was induced using blue light for either 60 or 90 seconds. After curing, 100 µL of 10% FBS-DMEM was added to each well and replaced every two days. Encapsulated cell viability was evaluated using the resazurin reduction assay on days 1, 3, 5, and 12. All experiments were performed in triplicate across three independent trials.

Evaluation of cell morphology

To evaluate L929 fibroblast cell morphology on BL-MeHA hydrogel, samples were collected on days 1, 3, 7, and 14 after cell seeding. Culture media were removed, and wells were washed twice with 1 mL of PBS. Cells were then fixed with 1 mL of 2.5% glutaraldehyde at 25°C for 4 hours, followed by two PBS washes (1 mL each, 5 minutes per wash). Samples were dehydrated sequentially with 500 µL of 25%, 50%, and 75% ethanol at 4°C for 5 minutes each. This was followed by three changes of 500 µL 100% ethanol at 4°C for 5 minutes each. Subsequently, 300 µL of hexamethyldisilazane (HMDS) was added to each well for 20 minutes, then removed. Samples were frozen at -20°C for 3 hours, then transferred to -80°C for 24 hours prior to lyophilization. After 24 hours of freeze-drying, the samples were examined using scanning electron microscopy (SEM) and analyzed using ImageJ software.

Data analysis

The results were presented as mean ± standard deviation (SD) and were subjected to statistical analysis using SPSS 25.0 software (SPSS Inc, Chicago, IL, USA). The absorbance values were assessed for normal distribution using the Shapiro-Wilk test. A Kruskal-Wallis and Dunn's multiple comparisons analysis of variance was conducted to assess the presence of significant differences between the groups. ($p \leq 0.05$)

Results

BL-MeHA synthesis

In this study, we synthesized 4 separate batches of MeHA to evaluate the degree of modifica-

tion using ^{1}H NMR spectroscopy. All 4 batches demonstrated a 100% degree of substitution, meeting the criteria established by Chaopanitcharoen *et al.*,[16] Figure 1 presented the spectra obtained from the ^{1}H NMR analysis.

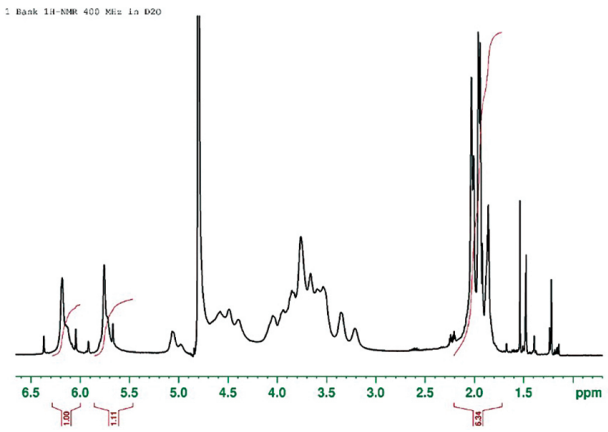

Figure 1: Degree of modification from proton nuclear magnetic resonance analysis.

Indirect cytotoxicity assay

After 24 hours of exposure to conditioned media derived from BL-MeHA hydrogel, the viability of L929 cells was assessed using the MTT assay as shown in Figure 2. The observed optical density (OD) values were 0.9371±0.0698, 0.9173±0.2137, 0.9208±0.0849, and 0.8736±0.0776 for the 100%, 50%, 25%, and 12.5% conditioned media groups, respectively. No statistically significant differences were found among these groups, indicating that the conditioned media was non-toxic to L929 cells. Furthermore, there was no significant difference when compared to 10% FBS-DMEM (0.9111±0.1015) control group. As expected, the 10% DMSO-treated group showed the lowest OD value at 0.4809±0.1143 which is significantly lower than all other groups.

2D culture assay

L929 cells were seeded onto the surface of the hydrogel and subsequently evaluated for viability using the PrestoBlue assay on days 1, 3, 5, and 12. The control group was conventional plastic cell culture plate. As shown in Figure 3, the control group exhibited the fastest growth starting from the 24-hour time point with the fluorescence value of 33,339.00±8,822.09, 37,684.33±11,611.77, 48,941.67±3,004.50 and 42,285.67±3,259.84 for day 1, 3, 5 and 12 respectively. In contrast, the L929 cells

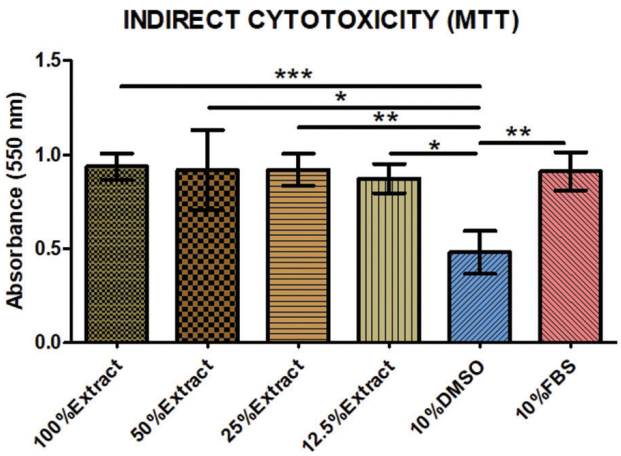

Figure 2: Indirect cytotoxicity assay (mean±SD, n=9, Kruskal-Wallis test and Dunn's multiple comparisons test, $p \leq 0.05$) *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

growing on top of the gel showed slower initial growth but were able to catch up with the control group by day 12. The fluorescence value for day 1, 3, 5 and 12 was 10,544.33±4,544.26, 13,210.00±2,090.41, 26,749.33 ±6,002.81 and 43,603.67±1,594.29 for the 60s BL-MeHA and 7,607.33±3,440.67, 22,667.66±6,270.75, 27,623.33±9,056.13 and 44,008.67±5,609.76 for the 90s BL-MeHA, respectively. No statistically significant difference was found between the 60s and 90s groups. Hydrogel without cell and blank media were included as negative controls, both showing very low reading.

Encapsulation culture assay

It was found that L929 were able to grow when encapsulated within BL-MeHA although the growth rate was slow. As shown in Figure 4, the relative fluorescence values on day 1, 3, 5, and 12 were 1.00±0.00, 5.84±2.76, 8.29±4.15 and 42.30±21.92 times for 60s BL-MeHA

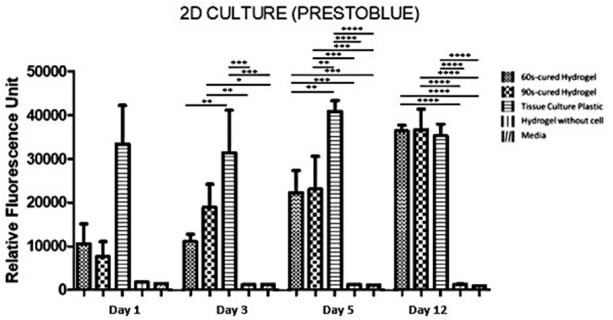

Figure 3: 2D culture assay (mean ± SD, n=9, One-way ANOVA and Tukey's multiple comparisons test, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ***$p \leq 0.001$)

and 1.00±0.00, 3.67±1.71, 5.23±3.09, and 33.18±13.39 for 90s BL-MeHA, respectively. For all time points, no statistically significant difference was found between the 60s and 90s groups.

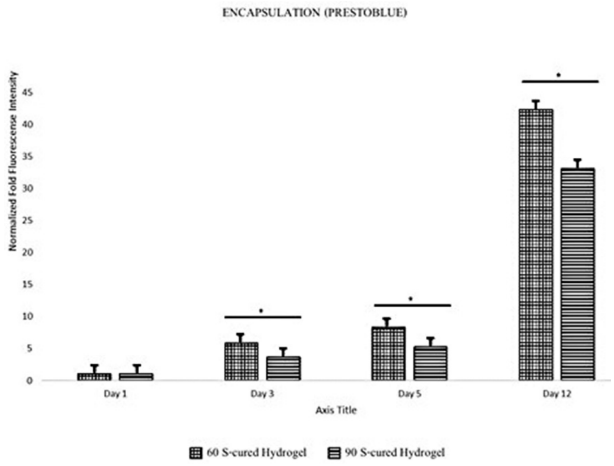

Figure 4: Encapsulation Culture Assay (mean±SD, n=9, Kruskal-Wallis test and Dunn's multiple comparisons test for intragroup analysis and Mann Whitney test for intergroup test, *$p \leq 0.05$).

Morphology assessment by scanning electron microscopy

On day 12 of the 2D culture assay, cell morphology was examined using scanning electron microscopy. As illustrated in Figure 5, both round and spindle-shaped L929 cells were observed, with some forming cellular clusters (A, B, C). Additionally, certain cells exhibited branching extensions and signs of cell division (I-arrow). These findings indicate that L929 cells were able to penetrate the hydrogel while maintaining their typical morphology and proliferative capacity within the hydrogel environment.

Discussion

Our group has been working on the development of hydrogel scaffolds for tissue engineering applications for some time. An earlier chemically crosslinked version showed promising physical and biological compatibility[13-15]; however, its prolonged setting time of approximately 30 minutes rendered it unsuitable for clinical use. The BL-MeHA hydrogel developed by Chaopanitcharoen *et al.,*[16] with significantly shorter curing times of 90 and 120 seconds, offers a practical solution to this limitation. In this study, we demonstrated that BL-MeHA not only possesses favorable physical properties but also exhibits excellent biocompatibility. The indirect cytotoxicity assay

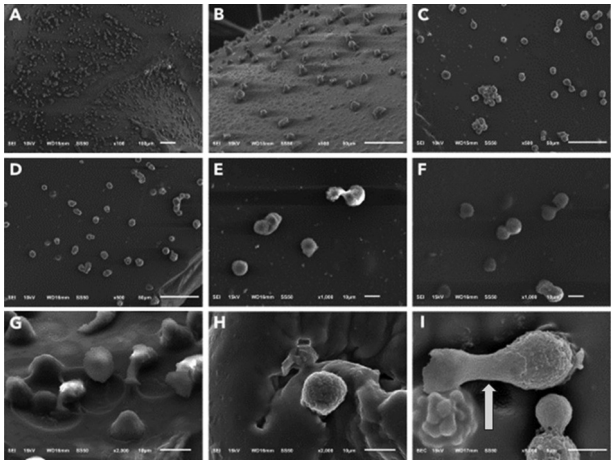

Figure 5: L929 morphology by scanning electron microscopy (2D culture assay day 12); (A) x100 and (B-D) 500X and 1000X(E-F) and 2000X (G-H) and 5000X (I) respectively.

demonstrated that L-929 cells remained morphologically normal after 24 hours of exposure to conditioned medium collected from BL-MeHA immediately after gel setting, even at the highest concentration of 100%, as well as at 50%, 25%, and 12.5%. This finding is promising for future applications, as the scaffold is intended to be in direct and prolonged contact with tissue. Interestingly, although the conditioned medium was serum-free, there was no statistically significant difference in L-929 cell growth compared to that observed with 10% FCS-DMEM. This may be attributed to the short 24-hour exposure period, during which the cells had limited time to proliferate. Nonetheless, the key finding is that L-929 cells remained viable, indicating that exposure to BL-MeHA conditioned medium is non-toxic.

L929 is an attachment-dependent cell line, requiring a surface for adherence in order to grow, survive and maintain its function.[17] In the 2D cell culture assay, L929 cells were able to grow on top of the BL-MeHA. Although cell numbers were significantly lower compared to those on a conventional cell culture plate at days 1, 3, and 5, the cells continued to proliferate and reached comparable levels by day 12. Additionally, there was no statistically significant difference in cell numbers between the 60-second and 90-second BL-MeHA groups, indicating that L929 cells were able to grow equally well on both gel types. Additionally, the encapsulation assay showed that L929 cells were able to survive and grow—albeit slowly—when embedded within the BL-MeHA hydrogel. This suggests that the gel permits sufficient diffusion of nutrients and

oxygen to support basic cellular viability. Once again, no significant difference was found between the 60s and 90s groups.

The use of blue-light activation to control the setting time has proven effective, offering a viable alternative to the chemically crosslinked method previously reported by Trakiattikul *et al.,*[13] While Chaopanitcharoen *et al.,*[16] demonstrated that curing durations of 90s and 120s produced optimal physical properties, the present study investigated shorter exposure times of 60s and 90s. These reduced durations were feasible due to the smaller volume of gel used, yet still yield satisfactory outcomes. Several factors can influence the required light-curing time, including the size and thickness of the gel, the intensity and quality of the light-curing units, and the presence of any materials that may obstruct light transmission.[18] It is therefore essential to determine appropriate curing time based on the specific conditions of each application. Nevertheless, the potential cytotoxic effects of direct LED light exposure should not be underestimated. Studies have demonstrated that intense violet or blue light can trigger photoreduction of flavins within mammalian cells, activating flavin-containing oxidases in mitochondria and peroxisomes, which in turn leads to the production of hydrogen peroxide (H_2O_2).[19] Additionally, LED curing lights have been shown to inhibit the proliferation of gingival epithelial cells and periodontal ligament fibroblasts, potentially causing damage to oral soft tissues.[20,21] Therefore, it is advisable to limit light exposure to the minimum duration necessary to achieve effective curing and reduce the risk of cytotoxicity.

In this study, the porous structure of the hydrogel is not visible in the SEM images due to the drying method used. The process involved gradually increasing ethanol concentrations to replace water in the sample, followed by final drying with hexamethyldisilazane (HMDS). While this method effectively preserves the cellular morphology close to its original state, it causes the hydrogel structure to collapse during drying, resulting in a less porous appearance under the electron microscope. This differs from the approach used by Chaopanitcharoen *et al.,*[16] where the hydrogel was dried before lyophilization, allowing better preservation of its porous architecture. However, lyophilization is not suitable for the current study, as it can damage animal cells, making it inappropriate for assessing cell-hydrogel interactions.

In conclusion, the results of this study demonstrate that the BL-MeHA hydrogel is biocompatible with the L929 cell line, as confirmed through indirect cytotoxicity, 2D culture, and cell encapsulation assays. These findings highlight the potential of BL-MeHA as a promising scaffold for the delivery of cells, growth factors, biomaterials, drugs, or other bioactive agents in future tissue engineering applications.

Conflicts of Interest

The authors declare no conflicts of interest.

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