THE BIOCOMPATIBILITY OF BLUE LIGHT ACTIVATED HYALURONIC ACID HYDROGEL WITH MOUSE OSTEOBLASTIC CELL LINE

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THE BIOCOMPATIBILITY OF BLUE LIGHT ACTIVATED HYALURONIC ACID HYDROGEL WITH MOUSE OSTEOBLASTIC CELL LINE

BY

PUTHITA LEEWISUTTHIKUL

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Bone plays a crucial role in the success of dental implant placement, while the use of bone grafting materials has encountered certain limitations and biomaterial as tissue engineering scaffold is of interest. This research aimed to assess the biocompatibility of blue light activated methacrylated hyaluronic acid (MeHA) hydrogel with MC3T3-E1 cell line which was designed for use as a drug delivery system. The hydrogel was synthesized from methacrylated hyaluronic acid (MeHA), cross-linked by LAP and activated with blue light. In vitro experiments were conducted to investigate the biological properties of the hydrogels, including cell proliferation and cell morphology in three-dimensionally cell culture. The results indicate that blue light activated MeHA hydrogel has no cytotoxic effects on MC3T3-E1 mouse osteoblastic cell line, as the cells remained viable throughout the 14-day culture period. Furthermore, scanning electron microscopy (SEM) analysis revealed normal cell surfaces with a round shape in general and some in a spindle shape. Therefore, the application of a blue light activated MeHA hydrogel, serving as both a both as a scaffold and drug delivery system for bone tissue engineering could be a promising approach.

Keyword : hyaluronic acid, hydrogel, tissue engineering, biocompatibility, MC3T3-E1 cell line

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PUTHITA LEEWISUTTHIKUL

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1
INTRODUCTION

Background

Nowadays, a dental implant is a choice of treatment and is considered an efficient option for tooth replacement. Alveolar bone resorption after tooth loss is unavoidable and compromises bone quality, quantity, and morphology which significantly complicates the subsequent dental implant placement. However, bone augmentation using grafting materials can improve the vertical and horizontal dimensions of the alveolar ridge for achieving a better esthetic outcome and allowing osseointegration. Osseointegration, defined as the direct structural and functional connection between the living bone and surface of a load-bearing implant (Branemark, 1983), is a critical factor for the long-term success of dental implants. Various types of bone grafting materials categorized by their original sources: autografts, allografts, xenografts, and alloplastic materials provide different degrees of bone formation and drawbacks. The disadvantages for example additional site of surgery, the risk of donor site complications, limited bone availability, uncertain resorption, the risk of disease transmission, and the presence of residual graft led to the study to develop alternative grafting materials for hard tissue engineering.

Tissue engineering involves basic and preclinical research and development on the repair, replacement, and regeneration of cells, tissues, or organs. It is composed of three fundamental elements: 1. cells 2. signaling molecules such as growth factors or cytokines 3. biomaterials or scaffolds for tissue regeneration.

Hydrogel, a hydrophilic three-dimensional polymer network, is extensively researched as a biomaterial for tissue engineering. They serve as scaffold providing structural integrity which allow cells to adhere, proliferate, and differentiate and use as drug delivery systems. Hydrogels can be synthesized from several different monomers. Hyaluronic acid-based hydrogel (HA hydrogel) has gained increasing interest due to its structural and compositional similarities to the extracellular matrix, biocompatibility, biodegradability.(1-4) Trakiattikul et al. and Areevijit et al. reported some limitations in developing hyaluronic acid-based hydrogel, for example, uncertain degree of

modification and uncontrolled gelation time.(5, 6) In 2021, Chaopanitcharoen et al. has developed methacrylated hyaluronic acid (MeHA) hydrogel activated by blue light to fasten the gelation time. They reported that at 90-seconds polymerization time, the hydrogel obtained suitable physical properties, consistent pore size, and swell ability.(7)

Recently, Jivacharoen et al. evaluated the biocompatibility of blue light activated MeHA hydrogel with L929 fibroblast, consisting of indirect cytotoxicity assay, proliferation in 2D and 3D culture assay. They reported that MeHA hydrogel was biocompatible with L929 fibroblast until day 12 of culture.(8)

For hard tissue engineering purposes, the aim of this study is to assess the biocompatibility of MeHA hydrogel activated by blue light with mouse osteoblastic cell line, MC3T3-E1.

Objective of the Study

To assess the biocompatibility of blue light activated MeHA hydrogel (90- and 120-seconds activation time) with MC3T3-E1 cell line.

1. Evaluate cell viability of MC3T3-E1 cell line cultured in 90- and 120-s blue light activated MeHA hydrogel.
2. Evaluate cell morphology of MC3T3-E1 cell line cultured in 90- and 120-s blue light activated MeHA hydrogel.

Significance of the Study

To develop blue light activated MeHA hydrogel which has suitable physical property and biocompatibility for application in hard tissue engineering.

Scope of the Study

In vitro study to develop blue light activated MeHA hydrogel which has suitable physical property for tissue engineering and biocompatibility with MC3T3-E1 cell line by evaluating cell viability using Resazurin assay and cell morphology using a scanning electron microscope (SEM).

Variables

Independent variable: Blue light activated MeHA hydrogel.

Dependent variable: The biocompatibility of blue light activated MeHA hydrogel with MC3T3-E1 cell line by evaluating cell viability using Resazurin assay and cell morphology using SEM.

Control variable: Minimum Essential Medium α (MEM-α)

Definition of terms

1. Blue light activated MeHA hydrogel: biodegradable biomaterial which is composed of methacrylated hyaluronic acid-based hydrogel activated by blue light.

2. MC3T3-E1 cell line: pre-osteoblast cell derived from the calvaria of a mouse embryo, is widely used for bone tissue engineering and in development of novel biomaterials in vitro.

Conceptual framework

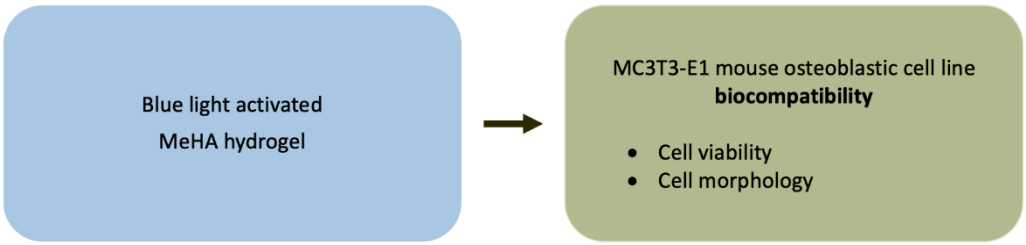


Figure 1 Conceptual Framework

Research hypothesis

Null hypothesis:

1. There is no difference between cell viability of MC3T3-E1 cell line cultured in 90- and 120-s blue light activated MeHA hydrogel.
2. There is no difference between cell morphology of MC3T3-E1 cell line cultured in 90- and 120-s blue light activated MeHA hydrogel.

Alternative hypothesis:

1. There is a difference between cell viability of MC3T3-E1 cell line cultured in 90- and 120-s blue light activated MeHA hydrogel.
2. There is a difference between cell morphology of MC3T3-E1 cell line cultured in 90- and 120-s blue light activated MeHA hydrogel.

CHAPTER 2
LITERATURE REVIEW

Related literature has been reviewed and discussed as listed:

Bone healing

Osteoblast cells

Tissue engineering

Hyaluronic acid-based hydrogel

Bone healing

Bone is composed of 70% inorganic material and 30% organic material approximately. Type I collagen is the main component (90%) of the organic matrix, the remaining 10% are composed of various proteins and carbohydrates such as glycosaminoglycans, proteoglycans, glycoproteins, and growth factors. The inorganic component of bone is mainly composed of phosphate and calcium ions, also contains bicarbonate, sodium, potassium, citrate, magnesium, carbonate, fluorite, zinc, barium, and strontium. The hydroxyapatite crystals are formed by the nucleation of calcium and phosphate ions. The collagen and mineral components of bone define its functional characteristics. The mineral components are in charge of the matrix compression strength, while the collagen fibers determine the tissue's elasticity and toughness.(9, 10) Natural bone is composed of cortical bone with a relatively high density where osteocytes are trapped within a mineralized extracellular matrix in haversian system and trabecular bone which is entirely porous. The outer and inner surfaces of cortical bone are covered by fibrous periosteum and endosteum, respectively. The bone repair process has 4 stages which are hematoma formation, fibrocartilaginous formation, bony callus formation, and the remodeling phase. Fracture disrupts the local tissue and vascular integrity, resulting in the formation of blood clots or hematoma. In the first 24 hours, the hematoma attracts polymorphonuclear neutrophils, monocytes, and macrophages to the site of

injury. The neutrophils secrete chemokines such as interleukin 6 (IL-6) and chemokine ligand 2 (CCL-2). Macrophages secrete inflammatory mediators, such as tumor necrosis factor-α (TNF-α), stromal-derived factor-1α (SDF-1α), fibroblast growth factors (FGFs), CCL-2, and bone morphogenetic proteins (BMPs), also attract mesenchymal stem cells and synovial fibroblasts from a variety of sources such as bone marrow, periosteum, endosteum, muscles, and blood vessels. Following the acute inflammatory phase, these stem cells and fibroblasts promote fracture healing. Granulation tissue providing progenitor cells and new vessels and disorganized ECM will form after the inflammatory phase because of hypoxia. Soft callus is formed in the late inflammatory phase, produced by chondrocytes and fibroblasts derived from the progenitor cells. Afterward, soft callus starts to undergo endochondral ossification and gradually replaced by the bony callus. During the remodeling phase, the bony callus eventually remodeled into a compact bone by osteoblasts and osteoclasts over the next several years.(11, 12)

Bone formation or osteogenesis can occur in two processes: endochondral ossification or intramembranous ossification. Both start when specialized mesenchymal stem cells (MSCs) condense. During endochondral ossification, MSCs differentiate into chondroblasts, begin to proliferate and produce a cartilage template. These cells produce collagen and proteoglycans to form ECM. Subsequently, chondroblasts differentiate into chondrocytes and produce alkaline phosphatase, which is an enzyme involved in mineralization process. Chondrocytes are then engulfed and resorbed by pre-osteoblasts, osteoblasts, and blood vessels and finally replaced by the bone marrow. In this phase, osteoclasts are involved to remodel the template and form bone marrow. The endochondral ossification is restricted to occur in long bones and at fracture sites.(13) On the contrary, intramembranous ossification occurs in non-long bones such as skull and clavicle. MSCs begin to proliferate and condense. Later, they differentiate into osteoprogenitor cells and osteoblasts, respectively. Osteoblasts produce type I collagen to form an ECM, some are embedded in matrix and finally differentiate into osteocytes. And the mineralization begins.(14)

Osteoblasts, derived from mesenchymal stem cells, are responsible for bone formation. As they produce the organic matrix through a process known as ossification or osteogenesis. They are responsive cells that produce osteoid, an unmineralized organic tissue that eventually undergo calcification. Osteocytes, derived from osteoblasts or bone-forming cells, occupy bone lacunae which can communicate with other osteocytes via extensive cellular processes, detect mechanical stress, and deliver mechanical stress-related signals for bone remodeling. Eventually, osteoclasts are macrophage-like cells which are responsible for bone resorption. In healthy people, bones are constantly remodeling.(15)

The main cells involved in bone remodeling are osteoblast and osteoclast. Although bone tissue heals spontaneously for most fractures, additional treatment is required for complete bone healing for complicated fractures and diseases. Various cytokines and growth factors recruit osteogenic progenitor cells which are involved in the bone healing process. When designing biomaterial scaffolds, the timing and concentration of the cytokines and growth factors are crucial concerns. Numerous growth factors play crucial roles in bone regeneration, such as bone morphogenic proteins (BMPs), transforming growth factor-beta (TGF-β), fibroblast growth factor (FGF), insulin-like growth factor (IGF), vascular growth factor (VEGF), platelet-derived growth factor (PDGF), and stromal-derived growth factor (SDF1). VEGF is a protein that controls endothelial cell proliferation. BMPs promote osteogenesis by stimulating mesenchymal stem cell differentiation into osteoblasts. The coadministration of VEGF and BMPs holds the potential for a synergistic impact, enhancing both angiogenesis and osteogenesis process. While VEGF potentially augment BMP-induced bone formation, FGFs are required in promoting angiogenesis by stimulating the proliferation of endothelial and osteoblast cells. Currently, for clinical applications in human, BMP-2 and BMP-7 have received approval. While BMP-2 and BMP-7 have demonstrated favorable results, it is essential to consider the potential for adverse reactions in humans due to the administration of high doses, inflammatory side effects, and short time release at defect sites. Moreover, growth factors play a crucial role in the recruitment of progenitor cells to

healing sites. It is critical to provide the necessary biological signal to promote bone formation once cells have been delivered. Engineered biomaterials have been developed to deliver cells and signals at the appropriate release time. (4, 13)

Osteoblast cell

Osteoblasts are characterized as mononucleated cuboidal cells responsible for synthesizing the bone matrix and participating in the mineralization process during bone formation. These osteoblasts differentiate from mesenchymal stem cells (MSCs), which possess the capability to differentiate not only into osteoblasts but also into chondrocytes, muscle cells, adipocytes, ligament cells, and tendon cells. The differentiation of MSCs into osteoblasts is facilitated by BMPs, which belong to the transforming growth factor-β (TGF-β) family. BMPs function as signaling molecules in fundamental biological processes, including but not limited to bone formation, neurogenesis, and the development of organs such as the kidney, gut, and teeth. Osteoprogenitor cells begin to express a variety of genetic markers when they differentiate into osteoblasts, for example, type collagen I which is crucial for subsequent mineralization of hydroxyapatite. Osteoblasts motivate calcium salts and phosphorous to precipitate and bond with organic bone matrix, to mineralize the bone tissue. Moreover, they produce an enzyme involved in bone mineralization, alkaline phosphatase (ALP), which can be detected during osteoblast differentiation. The high expression of ALP is related to the increasing osteoblasts differentiation. Osteoblasts express estrogen receptors which allow them to increase the number of osteoblasts and thus collagen production. When the bone formation is complete, osteoblasts become trapped within the matrix and differentiate into osteocytes, both play important roles in maintaining calcium level and bone remodeling. Some of them persist on the bone surface and differentiate into inactive bone-lining cells. (16)

Osteoblast cultures can be applied for a variety of purposes such as potential anabolic agents screening, new biomaterials testing and development, and tissue engineering, other than using to evaluate the biochemistry and physiology of bone

formation and the molecular and cellular basis of human bone disease. Osteoblast cell can be isolated from periosteum, endosteum and MSCs.(17)

For studying osteoblast cell biology, diverse cell culture models have been proposed. These encompass primary cell cultures obtained from various species such as human, mouse, rat, bovine, ovine, and rabbit, as well as differentiated osteoblasts generated from pluripotent stem cells and the utilization of immortalized and malignant cell lines. Different models present advantages and disadvantages. Osteosarcoma cell lines offer the advantage of being readily obtainable in significant quantities, circumventing the need for isolation procedures or ethical approvals. Moreover, they provide the added benefit of consistent reproducibility. On the other hand, primary cells exhibit a behavior that is closely with preclinical and clinical relevance. In contrast, owing to their inherent heterogeneity, they can exhibit variability in their characteristics and behaviors, making it challenging to achieve uniformity in experiments. Advantages of cell lines include straightforward maintenance, the accessibility of cells without the need for isolation, and the preservation of phenotypic stability.(18)

MC3T3-E1 cell line

The osteoblastic cell line MC3T3-E1 is a clonal cell line of immature osteoblasts derived from a C57BL/6 mouse calvaria. MC3T3-E1 cells have a fibroblast-like shape and the size is 20–50 μm in diameter.(19) MC3T3-E1 cell is one of the most used in bone tissue engineering and in osteogenic model system owing to its proliferation and differentiation properties, type I collagen synthesis and ALP enzyme activity since day 3 during culture. Calcified bone tissue formation has been shown in vitro and hydroxyapatite deposition has been identified in the presence of ascorbic acid and inorganic phosphate.(20, 21)

MC3T3-E1 is cultured in MEM-α culture medium supplemented with 10% FBS (v/v), 2 mM L-glutamine, penicillin/streptomycin (100 unit/mL penicillin-100 L/mL streptomycin). The passage number and different osteogenic culture supplements influence the osteogenic capacity of MC3T3-E1 cells, Yan et al. suggested that avoid using the cells after passage 30 and use the combination of ascorbic acid, β-

glycerophosphate and dexamethasone as the osteogenic induction of MC3T3-E1 cells.(20, 22)

Tissue engineering

Tissue engineering is a practice that arose from the field of biomaterials development, composed of three fundamental elements: scaffolds, cells, and bioactive molecules. The primary objective of tissue engineering is the creation of functional constructs designed to rehabilitate, sustain, or enhance damaged tissue or complete organs. Biomaterials are an important component of bone regeneration. The success of scaffolds depends on their overall characteristics, such as surface, pore size, and bulk properties. Biomaterials are ideally expected to meet specific criteria, including biocompatibility, a degradation rate commensurate with new tissue generation, and transformation into non-toxic constituents without eliciting an immune response. Bio-incompatibility may result in consequences like immune system activation, tumor formation, and inflammation. The gradual enzymatic breakdown of the material following its introduction into living organisms is referred to as biodegradability, with the aim of ensuring the material's transformation into non-toxic constituents and can be excreted by the body.(13, 23)

Cells are responsible for producing a matrix, like that of natural tissue, are necessary for the development of engineered tissue in vitro. The use of primary cells isolated from human in combination with scaffolds to generate tissue has contributed to the most success in this field.(24) Continued developments focus on the design of new biomaterials for various tissue engineering and regenerative medicine applications, in terms of structure and mechanical properties to mimic natural tissue. Furthermore, the developed biomaterials are expected to preserve the phenotype of cultured cells and deliver the optimal growth factors under control.(25)

Significant characteristics to consider when designing bone scaffolds for successful integration and support of bone regeneration are surface roughness, internal porosity, pore size, interconnectivity, degradation, mechanical properties, and biocompatibility. The scaffold microarchitecture is essential for the proper transfer of

nutrients and waste, angiogenesis, and tissue infiltration. The degradation rate should correlate with the rate of new bone formation and the scaffold should tolerate mechanical stress relating to the surrounding bone as well. However, the increase in porosity and degradation rates can compromise mechanical strength.(13)

Hyaluronic acid hydrogel (HA hydrogel)

Hydrogel is a hydrophilic 3D cross-linked polymer network which can absorb a large amount of water or biological fluids. Due to its biocompatibility, biodegradability, mimicking the properties of extracellular matrix (ECM), hydrogel has gained a great interest in tissue engineering. Hyaluronic acid (HA), an acidic non-sulfated glycosaminoglycans, is a component of the ECM in the human body that maintains the viscoelasticity of the ECM, supports cellular structure, and acts as a lubricant. Hydrogel can be synthesized from various materials, either natural or synthetic, for example, chitosan, alginate, hyaluronic acid, Polyethylene glycol, Polyacrylamide and HEMA (2-Hydroxyethyl methacrylate). HA hydrogels can mimic human tissue in terms of high-water content, porosity, and ability to transport oxygen, nutrients, and metabolic waste. They can also act as carriers for growth factors or cells and as drug-delivery systems. Moreover, they can be modified to improve physical properties to use as drug delivery systems.(4, 15, 26)

Pereira et al. has developed the injectable modified hyaluronic acid from hydrazone-crosslinked hydrogel to deliver BMP-2 and reported that the modified hydrogel was biocompatible and has released BMP-2 for 28 days, 86% of BMP-2 were detected. Hydrogels have been extensively researched to develop a preferable drug delivery system by modification of HA hydrogel or cross-linking agents.(15) In 2015, Maturavongsadit et al. has developed HA hydrogel which is modified by the addition of methacrylic anhydride and crosslinked by cysteine-inserted Tobacco mosaic virus (TMV) mutants (TMV1cys) and Dithiothreitol (DTT). They proposed that thiol-acrylate can effectively control polymerization time and is considered safe. And 47 kDa hyaluronic acid has the preferable swelling ratio and reaches the required equilibrium. The microstructure of TMV-Cys-HA revealed 20 - 100 μm of pore size, appropriate for cells or substances

penetration, mass transfer of nutrients and metabolites, and tissue growth. A faster gelation time was observed in TMV-Cys-HA hydrogels, comparing to MeHA hydrogels crosslinked by DTT. Subsequently, Maturavongsadit et al. monitored the effect of different types of crosslinkers, including dithiothreitol (DTT), 4-arm polyethylene glycol (PEG), and multi-arm polyamidoamine (PAMAM), on the in vitro chondrogenic differentiation of bone MSCs in HA hydrogels. They mentioned that crosslinking of methacrylated hyaluronic acid with DTT has the fastest gelation time of 60 minutes.(27, 28)

In 2018, Trakiattikul et al. developed the injectable methacrylate hyaluronic acid hydrogel with mannitol/ bovine serum albumin which has desirable physical property but has considered long gelation time, 30 minutes.(29) In addition, Areevijit et al. reported that MeHA hydrogel crosslinked with DTT is biocompatible with human alveolar bone cells. However, gelation time at the average of 15–30 minutes, is a limitation for clinical applicability.(6)

Crosslink

Hydrogels are synthesized by two different polymerization techniques: chemical and physical. Chemical cross-linking agents, for example, carbodiimides, carbonyl diimidazole, butanedioldiglycidyl ether (BDDE), divinyl sulfone, glutaraldehyde have been reported of their toxicity and undesired reactions. Physical crosslinking can be achieved by a variety of pH, temperature, ionic strength conditions, and physicochemical interactions.

To improve HA hydrogels properties, including mechanical properties, viscosity, solubility, degradation, and biologic properties, hyaluronic acid has been chemically and crosslinker-modified. For the design of hydrogels, various crosslinking techniques are used.(30, 31)

Photo-crosslink

Photo-crosslink is one of the important techniques that has gained attention in modifying hydrogel structure, due to controllable gelation time and reduced the risk of

alteration of protein or hydrogel properties, which happened in addition to chemical crosslinker.(32)

In 2021, Chaopanitcharoen et al. has developed MeHA hydrogel activated by blue light to control the gelation time. They reported that at 90- and 120-second activation time, the hydrogel has suitable physical properties, consistent pore size, and swell ability.(7)

CHAPTER 3
RESEARCH METHODOLOGY

In this study, the experiments were conducted in the following orders:

Sampling

Methodology

Data collection

Statistical analysis

Sampling

The tested cells

MC3T3-E1 cells, mouse osteoblastic cell line

Blue light activated MeHA hydrogel

This research used the blue light activated MeHA hydrogel synthesized with the technique of Chaopanitcharoen et al.(7) They evaluated the different gelation time of 100mg/L MeHA hydrogel polymer in 15mg/L of Lithium phenyl-2,4,6-trimethyl-benzoylphosphinate (LAP) to develop the suitable hydrogel in terms of the physical property and suggested activating the gels for 90 and 120 seconds.

Methodology

Blue light activated MeHA hydrogel synthesis

The blue light activated MeHA hydrogel was synthesized following a protocol by Chaopanitcharoen et al. (2021) (Figure 2). Dissolved 1% by weight 47-kDa HA in potassium phosphate buffer at pH $\geq$ 8 then methacrylic anhydride (Me, MW 154.16 g/mol) was added dropwise in a 1:10 molar ratio with HA at 4 °C. The temperature of reaction was maintained between 0-4 °C. To maintain basic conditions (pH 8-10), 5 M of sodium hydroxide was used. The crosslinking reaction was allowed to proceed for 24 hours at 4 °C on a magnetic stirrer.

Dialysis started after the overnight stirring, immersed SnakeSkin Dialysis Tubing volume 3.7mL/cm, 30 cm long in hot water for 2 mins and RT water for 10 mins.

MeHA solution was transferred into the tubing, tied the tip of both ends and attached to the floating device. Dialysis against ultrapure water (3 L) on a magnetic stirrer for 72 hours at 4 °C. Replaced the ultrapure water 10 times.

Afterward, the solution was subsequently centrifuged 10,000 r/min at 20 °C for 10 min. The supernatant was collected and flash frozen before lyophilization (Labconco lyophilizer, Missouri) for 3 days.

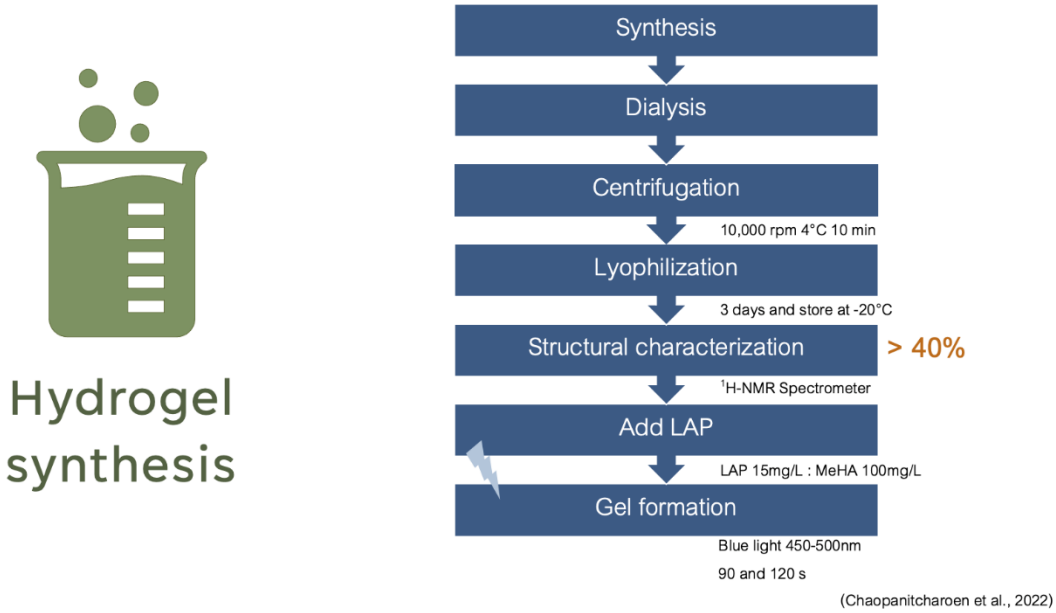


Figure 2 Protocol for blue light activated MeHA hydrogel synthesis

The degree of modification of hydrogel was analyzed by ^{1}H–NMR spectroscopy (Bruker, Rheinstetten, Germany), measuring from the absence of a double bond of methacrylate which indicates the formation of cross-linking hydrogels. (7) Before analysis, weighed 13 mg of MeHA polymer and dissolved in 0.5 ml Deuterium oxide (D_2O) in ^{1}H–NMR tube. The results were illustrated in Figure 3. The value obtained from the graph were calculated from Degree of modification $= \frac{a \times \frac{3}{2}}{c-(a \times \frac{3}{2})} \times 100$. Our previous study suggested that the degree of modification of hydrogel should be ≥ 40%.

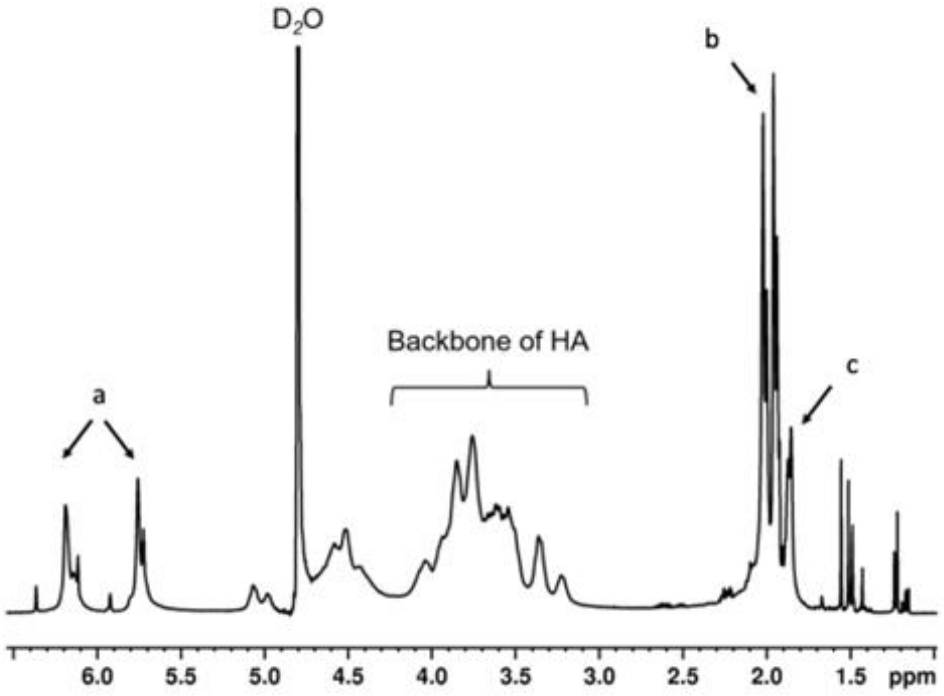


Figure 3 ^{1}H–NMR spectroscopy of MeHA hydrogel

Finally, the hydrogel was sterilized by soaking in 99.99% ethyl alcohol and irradiated under UV for 24 hours. Figure 4 demonstrated the synth5esis of MeHA hydrogel.

Before activating the pre-gel solution by blue light (R Max Cure 9, wavelength 480 nm, radiant intensity 1,800 mW/cm^{2}) at 1 cm away from the surface for 90 and 120 seconds, MeHA were dissolved in MEM-α supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 1% Penicillin 10,000 U/mL /Streptomycin 10,000 μg/mL. And LAP at a concentration of 15mg/L was added into 100mg/L MeHA polymer. The biocompatibility of MeHA hydrogel activated by blue light with MC3T3-E1 cell line was evaluated.

Figure 4 Methodology of blue light activated MeHA hydrogel synthesis

Biocompatibility evaluation of blue light activated MeHA hydrogel with MC3T3-E1 cell line

1. Cell culture

MC3t3-E1 Subclone 4 (ATCC CRL-2593), mouse pre-osteoblast cell, is from Department of Advanced General Dentistry, Faculty of Dentistry, Mahidol University. MC3T3-E1 cells were initially thawed from passage 6 and cultured in MEM-α culture medium supplemented with 10% FBS (v/v), 2 mM L-glutamine, penicillin/streptomycin (100 unit/mL penicillin-100 L/mL streptomycin). Cells were maintained at 37 °C in a reducing atmosphere (5% CO2). The medium was replaced every 2-3 days. Subcultures were performed at 70-80% confluency. Passage 10-15 were used in the study.

2. Seed MC3T3-E1 cells on blue light activated MeHA hydrogel

The 3D encapsulation of cells within the MeHA hydrogel which mimic the ECM in vivo was used to evaluate the biocompatibility of MC3T3-E1 cells with hydrogel. After lyophilization and sterilization by 99.99% ethyl alcohol, the prepared hydrogel was dissolved in MEM-α supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine

and 1% Penicillin 10,000 U/mL /Streptomycin 10,000 μg/mL at a concentration of 100 mg/L and LAP was added at a concentration of 15 mg/L. A volume of 50 μL of pre-gel solution was pipetted, then seeded with 1×10^4 MC3T3-E1 cells before gently pipetting up and down to create a uniform gel/cell suspension. Transferred a volume of pre-gel solution containing MC3T3-E1 cells into each well in 96-well plate. Blue light with a wavelength of 480 nm and a radiant intensity 1,800 mW/cm^2 was used to activate the gel for 90 and 120 seconds at 1 cm away from the surface. Finally, added 100 μL of MEM-α and incubated at 37 °C in a reducing atmosphere (5% CO2). The medium was replaced every 2-3 days.

For cell viability assay, cells evaluated at each time point were seeded on the same culture plate.

3. Cell viability assay

Evaluated cell viability using resazurin-reduction assay for 1, 3, 7, and 14 days after cell seeding. Resazurin (PrestoBlue™, cell viability reagent, Invitrogen; ThermoFisher Scientific) was prepared according to the manufacturer's instructions then added to all wells (10% PrestoBlue™), Following this, the plate was shaken for 30 seconds and incubated at 37 °C in a reducing atmosphere (5% CO_2) for 1 hour. Viable cells reduced resazurin to resorufin which is a fluorescent dye. Finally, the fluorescence intensity which is proportional to the number of cells was analyzed by Microplate reader (CLARIOStar, BMG Labtech, USA) at a wavelength of 560/590 nm. Experiments were conducted in technical triplicates (n = 3).

4. Evaluation of cell morphology

Evaluated osteoblast morphology after seeding on MeHA hydrogel activated by blue light at day 10. Discarded the medium from the plates and washed twice with 1 μL of PBS each well. Fixed with 1 ml 2.5% glutaraldehyde at 25 °C for 4 hours. Washed twice with 1 μL of PBS for 5 mins each time. Dehydrated with 500 μL of 25%, 50% and 75% ethanol at 4 °C for 5 mins, sequentially. Additionally, 500 μL of 100% ethanol was added at 4 °C for 5 mins thrice. Finally, added 300 μL hexamethyldisilazane (HMDS) to each well for 20 mins then discarded. After 24 hours, the samples were

examined using a scanning electron microscope (SEM) and processed with Image J program.

Data collection

1. Evaluated cell viability from fluorescence intensity at excitation/emission of 560/590 nm using resazurin-reduction assay.
2. Evaluated cell morphology with a scanning electron microscope (SEM).

Statistical analysis

Kruskal-Wallis test and Dunn's multiple comparisons test were carried out to compare the different groups. Mann-Whitney test was carried out to compare the different groups of cells at each time point. Statistical analyses were performed using GraphPad Prism Software 10. $P < 0.05$ was considered statistical significance. All experiments were conducted in technical triplicates ($n = 3$).

CHAPTER 4
RESULTS

This study evaluated the biocompatibility of MC3T3-E1 cell line with blue light activated MeHA hydrogel (90- and 120-seconds activation time). The results are presented as follows:

1. Hydrogel synthesis
2. Cell viability assay
3. Cell morphology assay

Cell viability was assessed by measuring fluorescence intensity at an excitation/emission wavelength of 560/590 nm using resazurin-reduction assay. The data are reported as mean ± standard deviation (SD), resulting from three or more replicates. Statistical analyses were performed due to abnormal distribution of data. The Kruskal-Wallis test and Dunn's multiple comparisons test were conducted to compare the different groups, while the Mann-Whitney test was used to compare the different groups of cells at each time point. All statistical analyses were performed using the GraphPad Prism Software with a p-value < 0.05.

Hydrogel synthesis

MeHA polymers were synthesized four times and presented with 64.8%, 30.01%, 44.33% and 58.19% modifications (Table 1), and analyzed by ^{1}H–NMR spectroscopy (Figure 4). All hydrogels which have the degree of modification $\geq$ 40%, were used for the tests in the study. Blue light initiator (LAP) at a concentration of 15mg/L was added into 100mg/L MeHA polymer. The biocompatibility of blue light activated MeHA hydrogel with MC3T3-E1 cell line was evaluated.

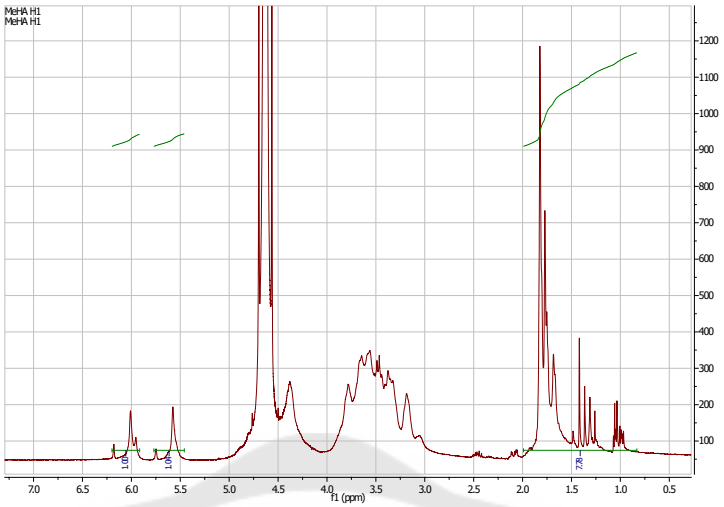


Figure 5 ^{1}H–NMR spectroscopy of MeHA hydrogel

Table 1 Degree of modification of MeHA hydrogel synthesized in the study.

Batch	Degree of modification (%)
1	64.8
2	30.01
3	44.33
4	58.19

Cell viability assay

Following the 3D encapsulation of MC3T3-E1 cells within the MeHA hydrogel, a cell viability assay using the resazurin assay was performed at day 1,3,7 and 14. The fluorescence intensity of MC3T3-E1 cells cultured in 90-s blue light activated hyaluronic acid hydrogel at day 1,3,7 and 14 were 3259±537.58, 2953±638.40, 4033.67±275.13 and 4385.33±1063.75 arbitrary units (AU), respectively. The fluorescence intensity of MC3T3-E1 cell line cultured in 120-s blue light activated hyaluronic acid hydrogel at day 1,3,7 and 14 were 3698.67±623.29, 3134.67±616.14, 3591.33±321.50 and 4324.33±1696.63 AU, respectively. According to the results, the metabolic rate of viable cells within the MeHA hydrogel gradually increased over the culture time of 14 days. And showed no significant

differences between MC3T3-E1 cell line cultured in 90- and 120-s blue light activated MeHA hydrogel. Figure 6 and 7 illustrated the proliferation assay during the culture time of 14 days.

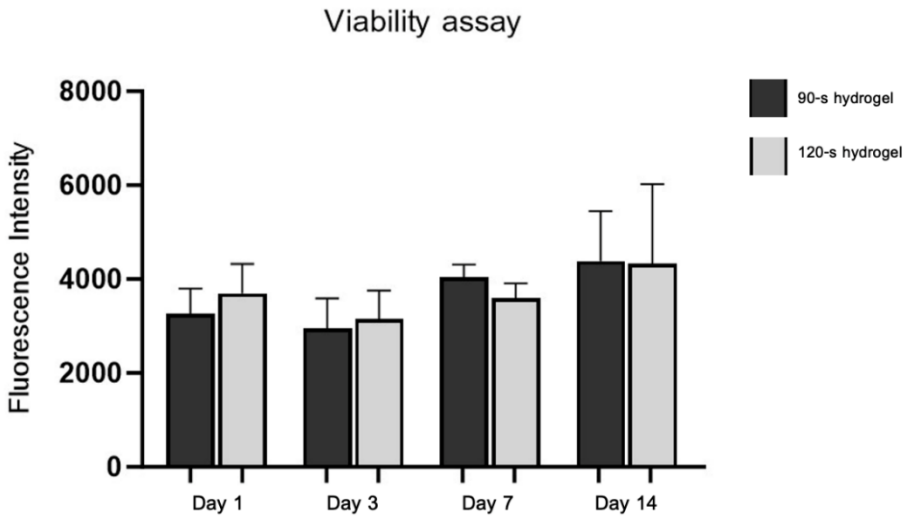


Figure 6 Bar graph represents proliferation assay, mean ± SD, using Kruskal-Wallis test and Dunn's multiple comparisons test to compare the different groups and Mann Whitney test to compare the different groups of cells at day 1, 3, 7, and 14, $p<0.05$

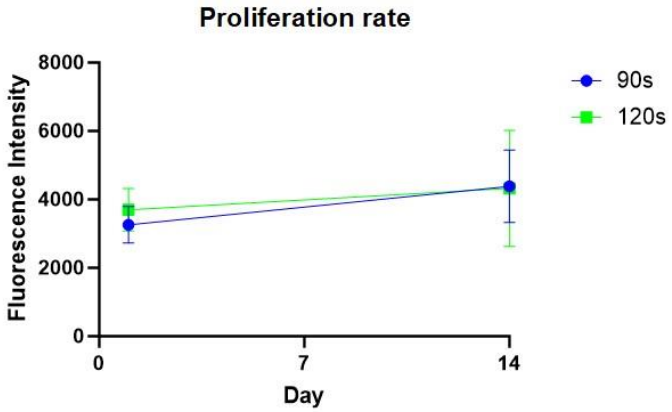
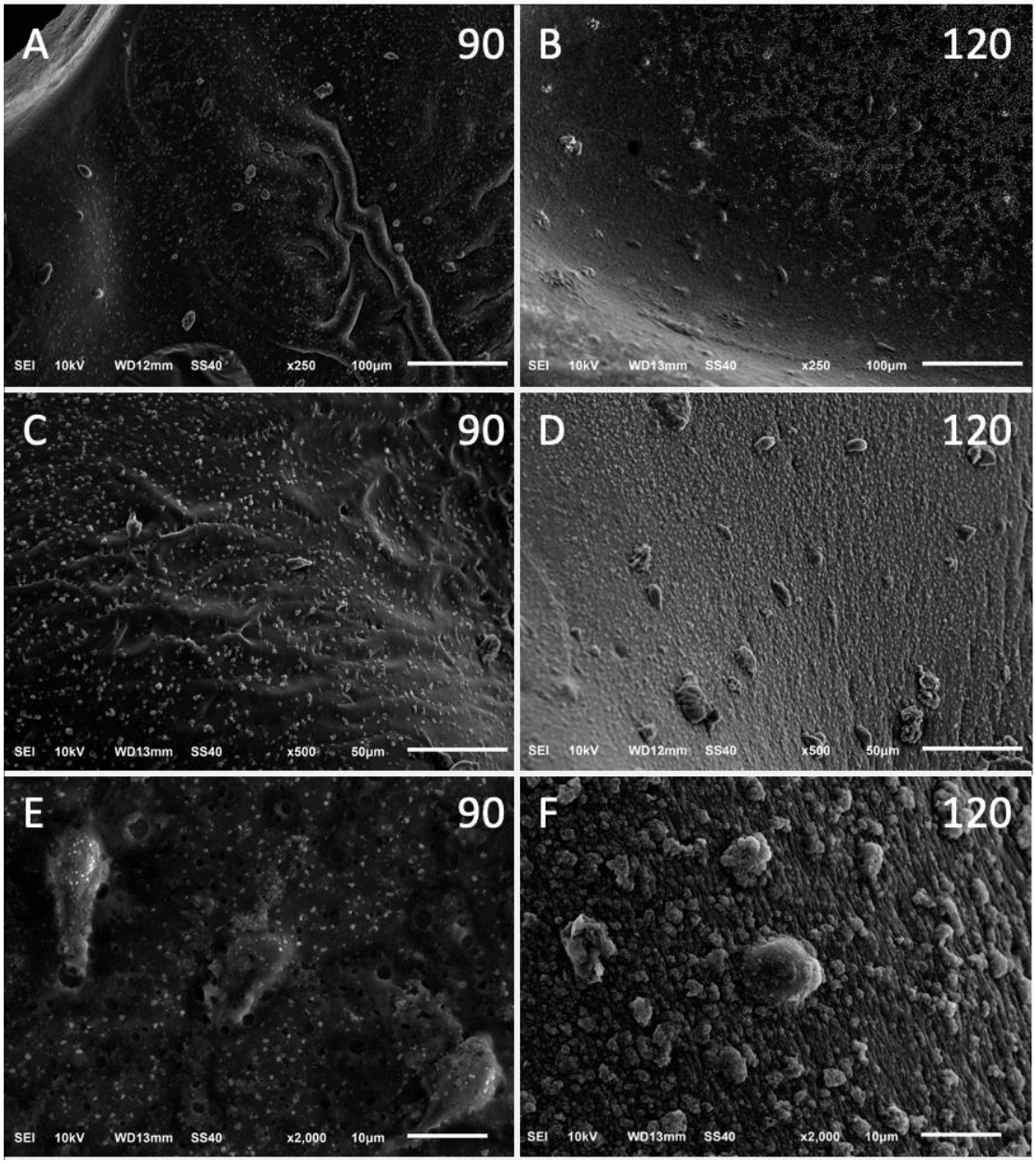


Figure 7 Dot graph represents proliferation rate, mean ± SD, using Kruskal-Wallis test and Dunn's multiple comparisons test to compare the different groups and Mann Whitney test to compare the different groups of cells at day 1 and 14, $p<0.05$

Cell morphology

Following the 3D encapsulation of MC3T3-E1 cells within the MeHA hydrogel, a cell morphology assay was performed on day 10 under SEM (Figure 8). The cell morphology exhibited round and spindle-shaped, with some cells appearing grouped together. These observations aligned with normal cell surface seen under inverted light microscope (data not shown). And there were no differences between cells cultured in 90- and 120-s blue light activated MeHA hydrogel (Figure 9).



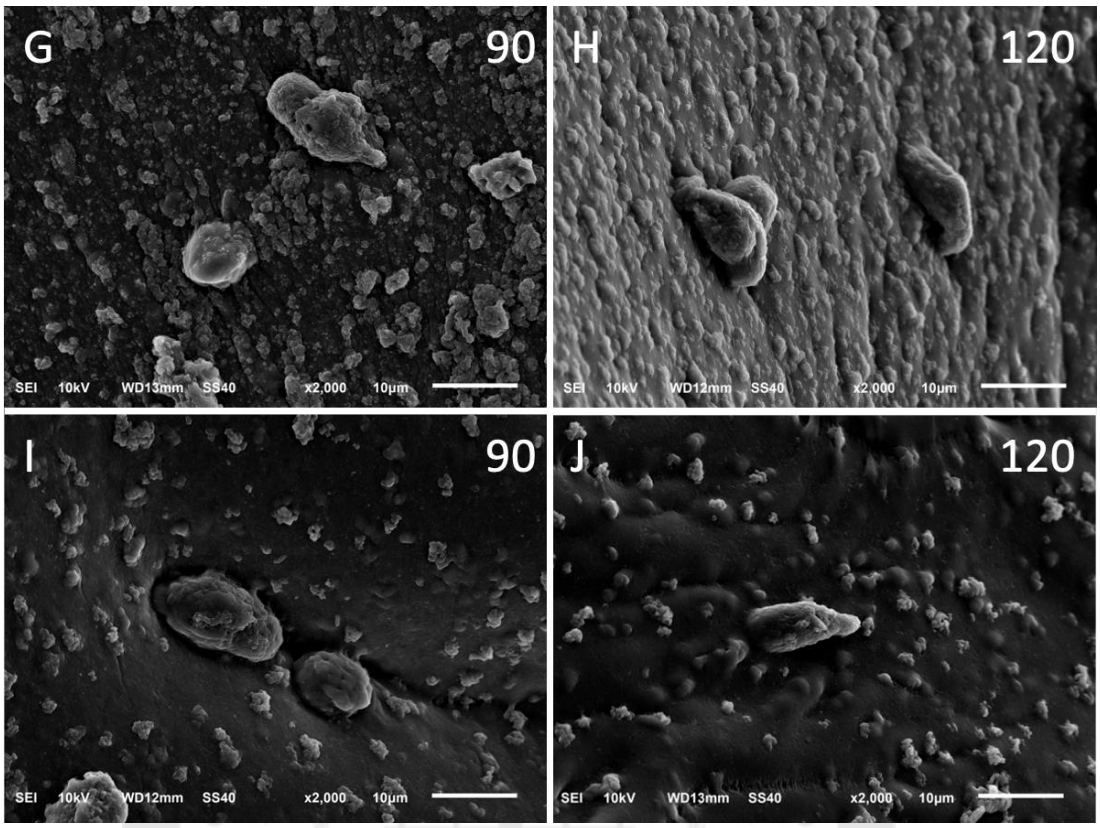


Figure 8 SEM images of morphology of MC3T3-E1 cells encapsulated in MeHA hydrogel at day 10: (A, C, E, G and I) Cells cultured is 90-s blue light activated MeHA hydrogel and (B, D, F, H and J) Cells cultured is 120-s blue light activated MeHA hydrogel.

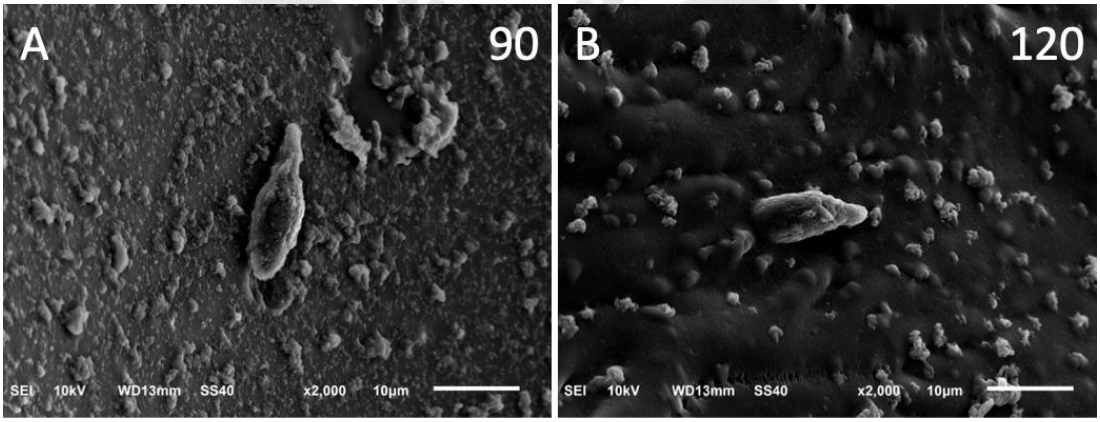


Figure 9 SEM images of morphology of MC3T3-E1 cells encapsulated in MeHA hydrogel after day 10 at 2,000X magnification: Comparing the cells cultured in 90- (A) and 120-s (B) blue light activated MeHA hydrogel.

CHAPTER 5
SUMMARY DISCUSSION AND SUGGESTIONS

This study evaluated the biocompatibility of mouse osteoblastic cell line MC3T3-E1 and blue light activated MeHA hydrogel. The results are summarized as listed:

1. Summary
2. Discussion
3. Suggestions

Summary

In this study, we assessed the biocompatibility of mouse osteoblastic cell line MC3T3-E1 with a blue light activated hyaluronic acid (HA) hydrogel, designed for use as a drug delivery system. The hydrogel was fabricated from methacrylated hyaluronic acid (MeHA), crosslinked with LAP, and activated using blue light. The synthesized MeHA hydrogels obtained the desired properties and were replicable. Both the 90- and 120-s blue light activated MeHA hydrogels demonstrated no cytotoxic effects on MC3T3-E1 mouse osteoblastic cell line. The cells remained viable throughout a 14-day culture period and exhibited normal cell surface with round and spindle-shaped cells when observed under SEM in 3D encapsulation. Therefore, this blue light activated MeHA hydrogel could be a promising drug delivery system for bone tissue engineering, offering favorable gelation time.

Discussion

HA hydrogel has been extensively researched as a biomaterial in tissue engineering due to its favorable characteristics, including biocompatibility with human tissues, biodegradability, mimic the properties of ECM, and adaptability to improve a physical property to serve as drug delivery systems. In a prior study by Chaopanitcharoen et al.(7), MeHA hydrogel crosslinked with LAP was developed. Using blue light to shorten the gelation time, comparing to the study by Areevijit et al.(6), to facilitate clinical

applications. In the present study, we evaluated the biocompatibility of blue light activated MeHA hydrogel with mouse osteoblastic cell line MC3T3-E1, consisting of cell proliferation and cell morphology evaluation in 3D encapsulation. We compared the cells cultured in 90- and 120-s blue light activated MeHA hydrogel, which hydrogel exhibits desirable physical properties, including consistent pore size, swell ability and susceptibility to degradation by hyaluronidase.

MC3T3-E1 Subclone 4 (ATCC CRL-2593) is a clonal cell line of immature osteoblasts derived from *Mus musculus* mouse calvaria. The cell grows as an adherent cell, has a fibroblast-like morphology, and is frequently employed in bone tissue engineering studies. Courtesy of Department of Advanced General Dentistry, Faculty of Dentistry, Mahidol University, MC3T3-E1 cells were thawed from passage 6 and cultured in MEM-α culture medium supplemented with 10% FBS (v/v), 2 mM L-glutamine, penicillin/streptomycin (100 unit/mL penicillin-100 L/mL streptomycin). The medium was replaced every 2-3 days. And cells were subcultured at 70-80% confluency. Passage 10-15 were used in the study.

MeHA polymers were synthesized four times and subsequently analyzed by ^{1}H–NMR spectroscopy. The synthesized MeHA polymer demonstrated reproducibility with a degree of modification ranging from 30.01 to 64.8%. This degree of modification was consistent with previous studies by by Chaopanitcharoen (7), Areevijit (6), Trakiattikul (29) and Maturavongsadit (27), which reported values of 42-76%, 42.03-66.37%, 21.8-41.38% and 40-50%, respectively. However, in contrast, Jivacharoen (8) reported a degree of modification of 100%. The synthesis of MeHA hydrogel is sensitive to various factors such as pH, temperature, and the timing of methacrylate anhydride addition. The reaction stops if the pH falls below 8 and temperature is not maintained at 0-4 °C, while polymer degradation occurs when pH exceeds 10. After LAP addition, MeHA hydrogels were dissolved in MEM-α culture medium and activated with blue light at a wavelength of 480 nm and a radiant intensity 1,800 mW/cm^2 at 1 cm from the surface. The pre-gel solution, 50-100 μL in volume, transformed into a gel within a similar time range to the previous studies (7, 8), taking 90 to 120 seconds, whereas Chaopanitcharoen et al. dissolved

MeHA hydrogels in phosphate buffer, and Jivacharoen et al. dissolved them in Dulbecco's Modified Eagle's Medium (DMEM).

For hard tissue engineering purposes, mouse osteoblastic cells MC3T3-E1 were cultured within the hydrogels in the 3D model, providing a more relevant environment that mimicked in vivo cell behavior. The study anticipated that MeHA hydrogels activated with different times of blue light exposure would be biocompatible and have no negative effects on mouse osteoblastic cells. The proliferation assay indicated a gradual increase in metabolic activity from day 1 to day 14, with no statistically significant differences. However, the encapsulated cells remained viable on day 14 of culture. In contrast, Jivacharoen et al. (8) evaluated the biocompatibility of blue light activated MeHA hydrogel with L929 fibroblast cells, which exhibited a superior proliferation rate. In a previous study, the influence of material stiffness on proliferation rate of periodontal ligament cells (PDLs) was discussed, and it was found that softer materials (6 kPa) resulted in poor PDL proliferation. They assumed that the elastic modulus of the hydrogels would not be adequately support cell proliferation.(33)

In a morphology assay conducted on day 10 of cell culture, cell morphology was in a round shape in general and some in a spindle shape, indicating limited attachment to the surface of MeHA hydrogel. Cell adhesion, spreading, growth, and differentiation can be influenced by cell density, material stiffness, and surface roughness. As discussed in a study by Zhang et al. (34), primary osteoblast cultured in polydimethylsiloxane substrates with varying stiffness. When grown on a rigid substrate ~134 kPa, the osteoblasts exhibited an expansive, polygonal morphology. In contrast, when cultured on a softer substrate ~1.4 kPa, the osteoblasts assumed a smaller, rounded shape. Additionally, cell binding affinity to MeHA is mentioned, with one of the binding sites being the hydroxyl group on MeHA polymer. Modified MeHA hydrogel could result in a lack of binding sites, affecting cell attachment and cell proliferation.(33) In conclusion, The study found no cytotoxic effects of blue light activated MeHA on MC3T3-E1 cell growth.

The current study demonstrated that MeHA hydrogel has no cytotoxic on mouse osteoblastic cell line in in vitro model with a suitable gelation time, indicating the potential use of this system as a medical device to support the regeneration of hard tissues in dental treatment.

Suggestions

Additionally, growth factors are viable candidates for their capacity to regulate proliferation and differentiation of cells. The incorporation of hydrogels with signaling molecules like growth factors has the potential to enhance tissue regeneration. Further, this approach could be translated into an in vivo model.

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Appendix

Research plan

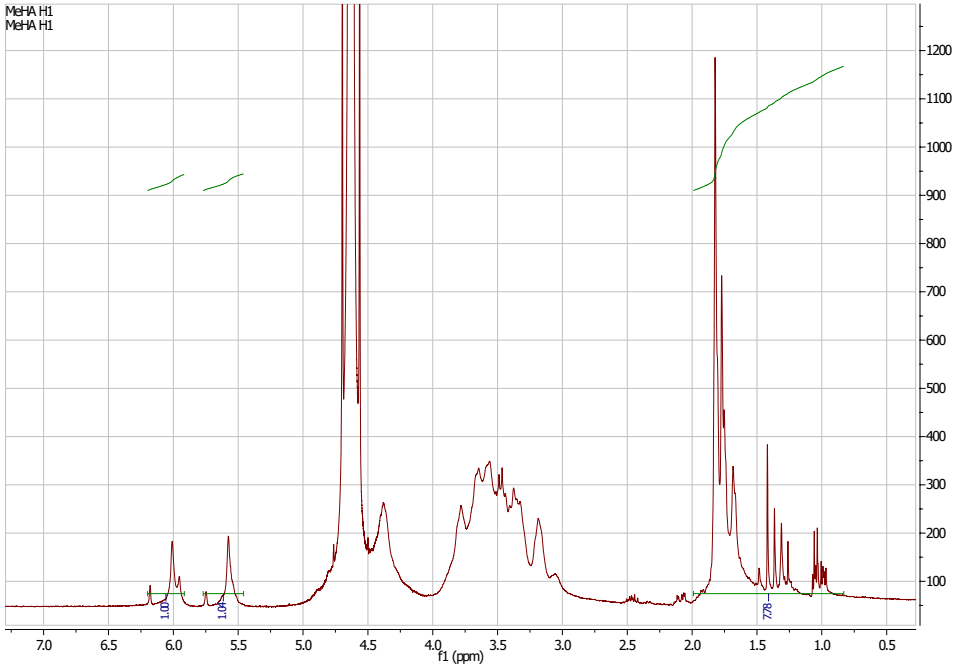
Method	Month											
	1	2	3	4	5	6	7	8	9	10	11	12
Advisor meeting and research topic	/											
Literature review and present research proposal		/	/	/								
Hydrogel synthesis -NMR test MC3T3-E1 cell culture					/	/	/					
Biocompatibility evaluation - Resazurin-reduction assay - Scanning electron microscope (SEM)								/	/			
Analysis and writing research results										/	/	/

Materials

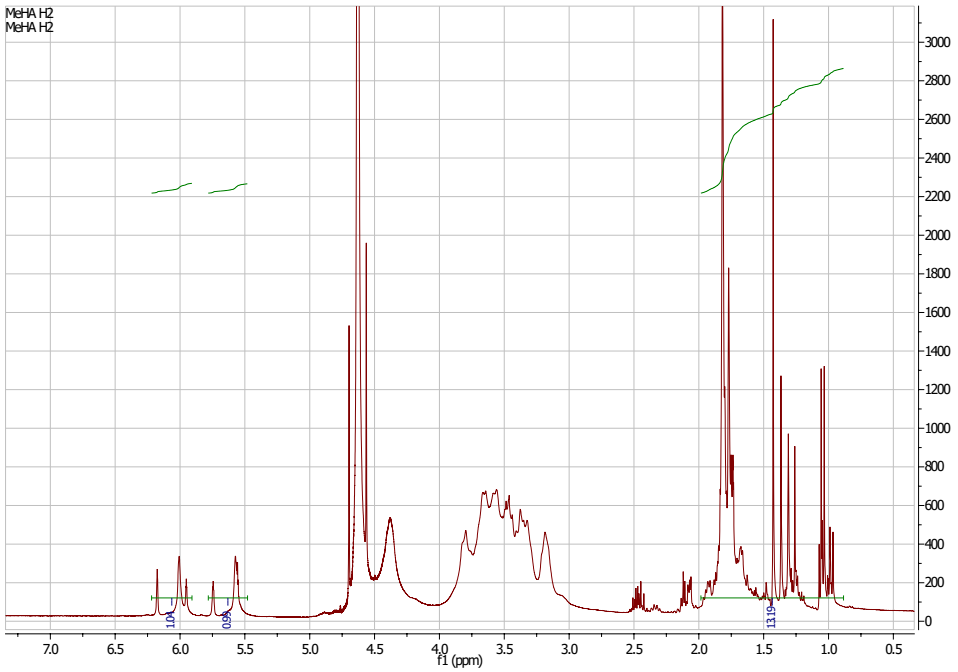
1. 47-kDa Sodium hyaluronate (Liuzhou Shengqiang Biotech Co., Ltd., Guangxi, China)
2. 0.1 M Potassium phosphate buffer
3. Methacrylic anhydride (Sigma Aldrich, USA)
4. 5 M Sodium hydroxide (Merck, Darmstadt, Germany)
5. Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP)
6. Deuterium oxide (D_2O) (Merck, Darmstadt, Germany)
7. Minimum Essential Medium-α (MEM-α) (Gibco, USA)
8. Fetal bovine serum (FBS) (Gibco, USA)
9. L-glutamine (Gibco, USA)
10. Penicillin/Streptomycin 10,000 U/mL (Gibco, USA)
11. Ultrapure water
12. Thermometer
13. pH indiaator
14. Ice
15. Magnetic stirrer (MSH-300, Biosan)
16. SnakeSkin Dialysis Tubing (ThermoFisher Scientific)
17. Centrifuge (Rotina 380 R, Hettich)
18. Lyophilizer (Labconco, Missouri)
19. Dental curing light (R Max Cure 9)

Degree of modification of MeHA hydrogel in the study

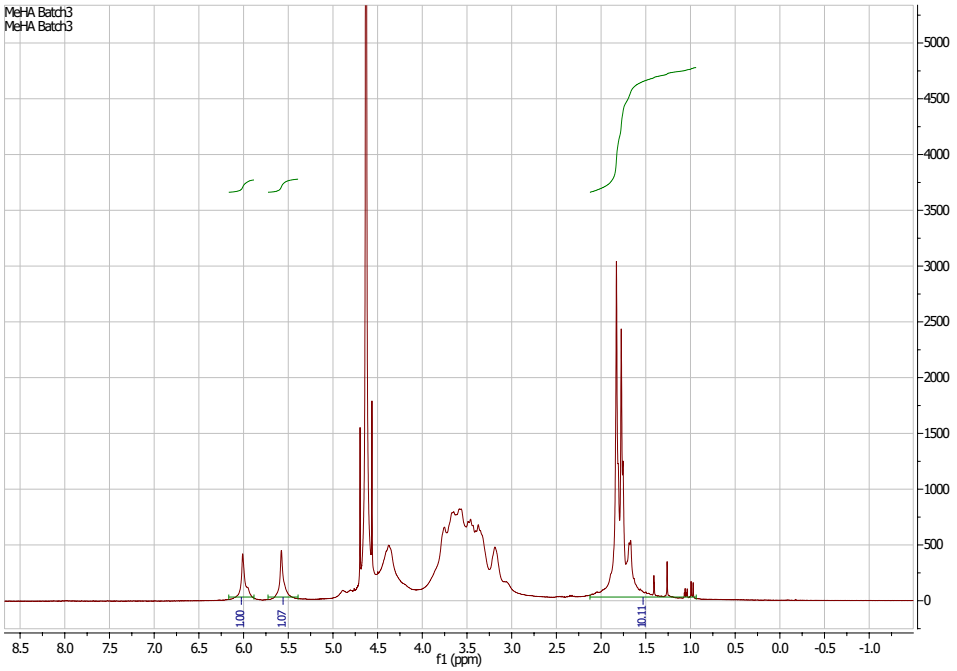
Batch 1: Degree of modification = 64.8%



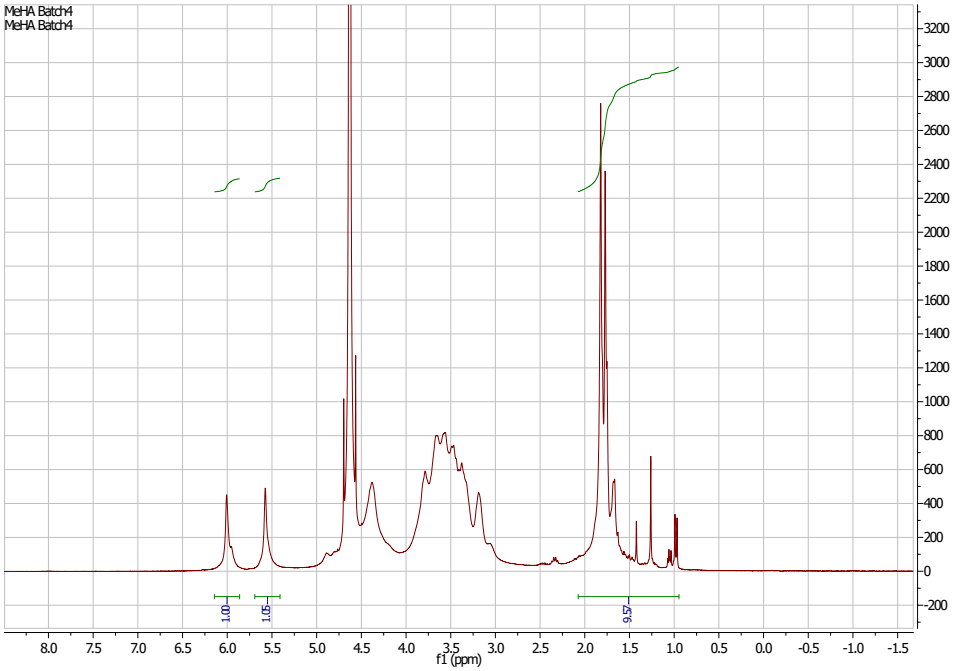
Batch 2: Degree of modification = 30.01%



Batch 3: Degree of modification = 44.33%



Batch 4: Degree of modification = 58.19%



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หนังสือแจ้งผลการพิจารณา
คณะกรรมการจริยธรรมสำหรับพิจารณาโครงการวิจัยที่ทำในมนุษย์ มหาวิทยาลัยศรีนครินทรวิโรฒ

เอกสารนี้เพื่อแสดงว่าคณะกรรมการจริยธรรมสำหรับพิจารณาโครงการวิจัยที่ทำในมนุษย์ ได้พิจารณาและตอบรับเอกสารเพิ่มเติมที่เกี่ยวข้องกับโครงการวิจัยดังนี้

ชื่อโครงการวิจัย : ความเข้ากันได้ทางชีวภาพของไฮโดรเจลจากกรดไฮยาลูโรนิกที่มีการกระตุ้นโดยแสงสีฟ้ากับเซลล์ออสติโอบลาสต์จากหนู

ชื่อหัวหน้าโครงการ : รองศาสตราจารย์ ดร. ทันตแพทย์สรสัณห์ รังสิยานนท์

หน่วยงานต้นสังกัด : คณะทันตแพทยศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ

หมายเลขรหัสโครงการ : SWUEC-097/2565X

เอกสารที่เสนอ : รายงานส่วนแก้ไขเพิ่มเติมโครงการวิจัย ฉบับลงวันที่ 27 กันยายน 2565

วันที่ประชุม : 21 พฤศจิกายน 2565 **การประชุมครั้งที่** : 11/2565

ผลการพิจารณา : รับรอง

วันที่รับรอง : 14 พฤศจิกายน 2565

ข้อเสนอแนะ:

- โปรดปฏิบัติตามแนวปฏิบัติการดำเนินงานโครงการวิจัยในมนุษย์ช่วงที่มีการระบาดของโรคติดเชื้อไวรัสโคโรนาสายพันธุ์ใหม่ 2019 (COVID-19)

ตอบรับโดย : คณะกรรมการจริยธรรมสำหรับพิจารณาโครงการวิจัยที่ทำในมนุษย์ มหาวิทยาลัยศรีนครินทรวิโรฒ

คณะกรรมการจริยธรรมสำหรับพิจารณาโครงการวิจัยที่ทำในมนุษย์ มหาวิทยาลัยศรีนครินทรวิโรฒ ดำเนินการให้การรับรองโครงการวิจัยตามแนวทางหลักจริยธรรมการวิจัยในมนุษย์ที่มีความสอดคล้องกับหลักจริยธรรมสากล ได้แก่ The Declaration of Helsinki, The Belmont report, CIOMS Guidelines และ The international Conference on Harmonization in Good Clinical Practice (IGH-GCP) ตลอดจนกฎหมาย ข้อบังคับและข้อกำหนดภายในประเทศ

(ลงชื่อ).....
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วันที่ : 21/11/2565

VITA

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