THE BIOCOMPATIBILITY OF BLUE LIGHT ACTIVATED METHACRYLATED HYALURONIC ACID HYDROGEL WITH MC3T3-E1 MOUSE OSTEOBLASTIC CELL LINE

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ABSTRACT

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 one of interest. This study aimed to evaluate the biocompatibility of MC3T3-E1 cell line and blue light activated hyaluronic acid (HA) hydrogel which was designed to be used as scaffold. The hydrogel was fabricated from methacrylated hyaluronic acid (MeHA) cross-linked by LAP and activated by blue light. The biological properties of hydrogels were performed in vitro model, consisted of proliferation in three-dimensionally encapsulated cell culture. The results revealed that blue light activated HA hydrogel was found to be biocompatible with mouse osteoblastic cell line (MC3T3-E1 cell). Therefore, the utilization of a blue light activated HA hydrogel both as a scaffold and drug delivery system for bone tissue engineering could be a promising approach. In addition, this could be translated into in vivo model for further investigation.

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

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INTRODUCTION

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 alloplasts provide different degrees of bone formation and drawbacks. The disadvantages for example additional site of surgery, the risk of donor site complications and limited bone availability, uncertain resorption, the risk of disease transmission, and the presence of residual graft lead 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. Hydrogels can be synthesized from several different monomers. HA-based hydrogel has gained increasing interest due to its structural and compositional similarities to the extracellular matrix, biocompatibility, biodegradability (Chircov, Grumezescu, & Bejenaru, 2018; Gao, Zhang, Zhang, & Zhang, 2016; Slaughter, Khurshid, Fisher, Khademhosseini, & Peppas, 2009). Hyaluronic acid (HA), acidic non-sulfated glycosaminoglycans, is a component of the extracellular matrix (ECM) in the human body that maintains the viscoelasticity of the ECM, supports cellular structure, and act 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-based 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 a physical property to use as drug delivery systems (Agarwal & García, 2015; Alvarez-Lorenzo & Concheiro, 2008; Pereira, Rodrigues, Rodrigues, Oliveira, & Gama, 2017).

Pereira et al. (2017) has proposed the injectable modified HA 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 MBP-2 were detected. Hydrogels have been extensively researched to develop a preferable drug delivery system by modification of HA-based hydrogel or cross-linking agents. Trakiattikul et al. (2018) developed the injectable methacrylate HA hydrogel with mannitol/ bovine serum albumin which has desirable physical property but has considered long gelation time, 30 minutes. In agreement with the study of Areevijit et al. (2019), MeHA hydrogel crosslinked with dithiothreitol (DTT) is biocompatible with human alveolar bone cells. Gelation time, in average 15–30 minutes, is a limitation for clinical applicability.

Chaopanitcharoen et al. (2021) has developed HA hydrogel polymerized by blue light to improve the gelation time. They reported that at 90- and 120-seconds polymerization time, the hydrogel has the suitable physical properties, consistent pore size, and swell ability.

Furthermore, Jivacharoen et al. (2022) evaluated the biocompatibility of blue light activated MeHA hydrogel with L929 fibroblast, consisted 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.
For hard tissue engineering purposes, the aim of this study is to evaluate the biocompatibility of mouse osteoblastic cell line, MC3T3-E1, and HA hydrogel activated by blue light.

LITERATURE REVIEWS

Bone healing

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 is involved in the bone healing process. When designing biomaterial scaffolds, the timing and concentration of the cytokines and growth factors are crucial concerns. Various growth factors involved in bone repair, including 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 to osteoblasts. The combination of VEGF and BMPs could provide a synergistic effect to promote angiogenesis and osteogenesis, as the VEGF potentially improve BMP-induced bone formation. FGFs are required for angiogenesis as they promote endothelial and osteoblast cell proliferation. Currently, BMP-2 and BMP-7 are approved for clinical use in human. Although the use of BMP-2 and BMP-7 has yielded desired outcomes, there are considerations about the possible adverse reactions in human owing to high dose applications, inflammatory side effects, and short time release at defect sites. Moreover, progenitor cells can be carried to promote healing at the 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 released time (Agarwal & García, 2015; Perić Kačarević et al., 2019).

Osteoblast cell

Osteoblasts are mononucleated cuboid cells which generate bone matrix and involved in mineralization in bone formation process. Osteoblasts are differentiated from mesenchymal stem cells (MSCs), which can also differentiate into chondrocytes, muscle, fat, ligament, and tendon cells.
For studying osteoblast cell biology, various cell culture models have been proposed, including primary cells from different species (human, mouse, rat, bovine, ovine, and rabbit), differentiated osteoblasts from pluripotent stem cells, immortalized and malignant cell lines. Different models present advantages and disadvantages. For example, osteosarcoma cell lines can be obtained in a large quantity without isolation or ethical approval, with the additional benefit of consistent reproducibility. Primary cells have an interesting feature in terms of comprising behavior which has stronger preclinical and clinical applicability. Advantages of cell lines are easy maintenance, availability of cells without isolation, and phenotypic stability (Czekanska, Stoddart, Richards, & Hayes, 2012).

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 (Gibon et al., 2012). 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 (Tevlek, Odabas, Çelik, & Aydin, 2018; Warita, Aoki, Kitamura, Shibuya, & Hosaka, 2019).

Tissue engineering

Tissue engineering is a practice that arose from the field of biomaterials development, composed of three fundamental elements: scaffolds, cells, and biologically active molecules. The goal of tissue engineering is to establish functional constructs that restore, maintain, or improve injured tissue or entire 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. Ideal criteria for biomaterial are biocompatible, degrade at the same rate as new tissue formation, and degrade into non-toxic components while not arousing immune response. Immune system activation, tumor formation, and inflammation are consequences of bio-incompatibility. The enzymatic degradation of the material gradually when installed into living organisms is referred as biodegradability. The material should be degraded into non-toxic components that can be excreted by the body (Koons, Diba, & Mikos, 2020; Perić Kačarević et al., 2019).

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, including porosity, pore size, and pore interconnectivity. 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. Meanwhile, the increase in porosity and degradation rates can compromise mechanical strength (Perić Kačarević et al., 2019).

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, mimic the properties of extracellular matrix, the hydrogel has gained a great interest in tissue engineering. Hyaluronic acid (HA), acidic non-sulfated glycosaminoglycans, is a component of the extracellular matrix (ECM) in the human body that maintains the viscoelasticity of the ECM, supports cellular structure, and act 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). Hyaluronic acid-based 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 a physical property to use as drug delivery systems (Agarwal & García, 2015; Alvarez-Lorenzo & Concheiro, 2008; Pereira et al., 2017).

Pereira et al. (2017) has proposed 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 MBP-2 were detected. Hydrogels have been extensively researched to develop a preferable drug delivery system by modification of hyaluronic acid-based hydrogel or cross-linking agents. Maturavongsadit et al. (2016) has developed hyaluronic acid-based 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 both TMV-Cys-HA and hyaluronic acid-based hydrogels revealed 20 - 100 μm of pore size, appropriate for cells or substances penetration, mass transfer of nutrients and metabolites, and tissue growth. In contrast, a faster gelation time was observed TMV-Cys-HA hydrogels. Subsequently,

Maturavongsadit et al. (2017) 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 hyaluronic acid-based hydrogels. They mentioned that crosslinking of methacrylated hyaluronic acid with DTT has the fastest gelation time of 60 minutes.

Also, Trakiattikul et al. (2018) 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. In agreement with the study of Areevijit et al. (2019), it revealed that methacrylate hyaluronic acid hydrogel crosslinked with DTT is biocompatible with human alveolar bone cells. Gelation time, in average 15–30 minutes, is a limitation for clinical applicability.

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 (Mane, Ponrathnam, & Chavan, 2016).

Chaopanitcharoen et al. (2021) has developed hyaluronic acid-based hydrogel polymerized by blue light to control the gelation time. They reported that at 90- and 120-seconds activation time, the hydrogel has the suitable physical property, consistent pore size, and swell ability. The aim of this study is to evaluate the biocompatibility of of MC3T3

RESEARCH METHODOLOGY

Blue light activated HA hydrogel fabrication

Fabricated the blue light activated HA hydrogel with the technique of Chaopanitcharoen et al. (2021) Dissolved 1% by weight 47-kDa HA in potassium phosphate buffer at pH ≥ 8 then methacrylic anhydride (Me, MW 154.16 g/mol) was added in a 1:10 molar ratio with HA at 4 °C and the temperature of reaction was maintained at 0-4 °C. The pH was adjusted to 8 by the addition of 5 molars of sodium hydroxide and the crosslinking reaction continued for 24 hours at 4°C. 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 for 3 days. Our previous study (Chaopanitcharoen, Dhanesuan, Luckanagul, & Rungsiyanont, 2021) suggested that the degree of modification of hydrogel should be ≥ 40%, analyzed by ^{1}H–NMR spectroscopy measuring from the absence of a double bond of methacrylate which indicates the formation of cross-linking hydrogels. Finally, the hydrogel was sterilized by soaking in 99.99% ethyl alcohol.

Before polymerizing the prepared hydrogel by blue light for 90 and 120 seconds, lithium phenyl-2, 4, 6-trimethyl-benzoylphosphinate (LAP) at a concentration of 15mg/L will be added into 100mg/L MeHA hydrogel polymer. The biocompatibility of MC3T3-E1 cell line and blue light activated hyaluronic acid hydrogel was evaluated.

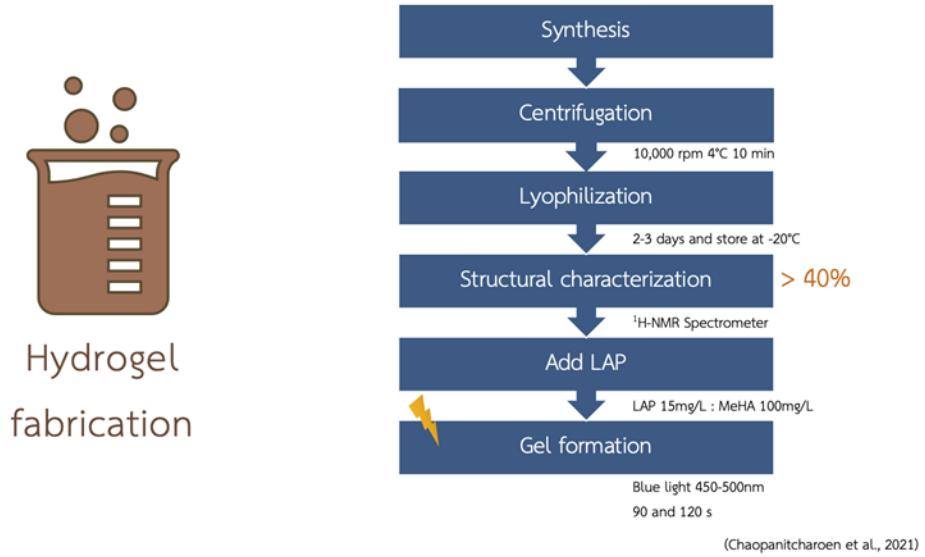

Figure 1 Blue light activated hyaluronic acid hydrogel fabrication

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

The 3D encapsulation of cells in a hydrogel which mimic the extracellular matrix 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 immersed 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 100mg/L and LAP was added at a concentration of 15mg/L. 50 µL of pre-gel solution was pipetted and seeded with 1×10^4 MC3T3-E1 cells before gently pipetting up and down to create a homogeneous gel/cell suspension. Transferred a volume of pre-gel solution containing MC3T3-E1 cells into each well in 96-well plate. Blue light (wavelength of 400-500nm) was used to polymerize the gel for 90 and 120 seconds to form a hydrogel. Finally, added 100 µL MEM-α and incubated at 37 °C in a reducing atmosphere (5% CO_2). The medium was replaced every 2-3 days.

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).

Statistical Analysis

Results were reported as mean ± standard deviation (SD), resulting from three or more replications. Kruskal-Wallis test was carried out to compare the different groups and Mann-Whitney test was carried out to compare the different groups of cells at each time point using the GraphPad Prism Software with a p-value < 0.05.

RESEARCH RESULTS

Hydrogel fabrication

HA polymers were fabricated four times and presented with 64.8%, 30.01%, 44.33% and 58.19% modifications, analyzed by ^{1}H–NMR spectroscopy (Figure 2). All hydrogels excluding one were used for the tests in the studies.

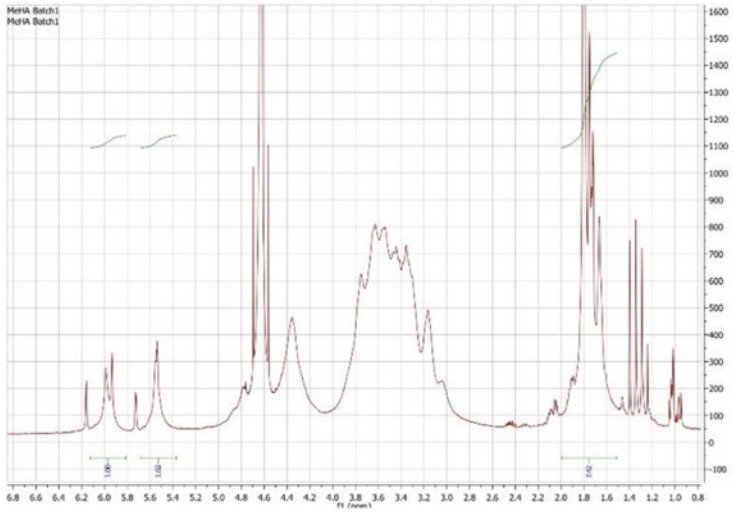

Figure 2 Degree of modification by ^{1}H-NMR Spectroscopy

Cell viability assay

After the 3D encapsulation of MC3T3-E1 cells in hydrogel, cell viability using resazurin assay was performed at day 1, 3, 7 and 14. Fluorescence intensity of MC3T3-E1 cell line 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, respectively. 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, respectively. According to the results, the metabolic rate of viable cells in MeHA hydrogel was slowly increased to day 14. And showed no significant differences between MC3T3-E1 cell line cultured in 90- and 120-s blue light activated hyaluronic acid hydrogel. Figure 3 illustrated the proliferation assay during the culture time of 14 days.

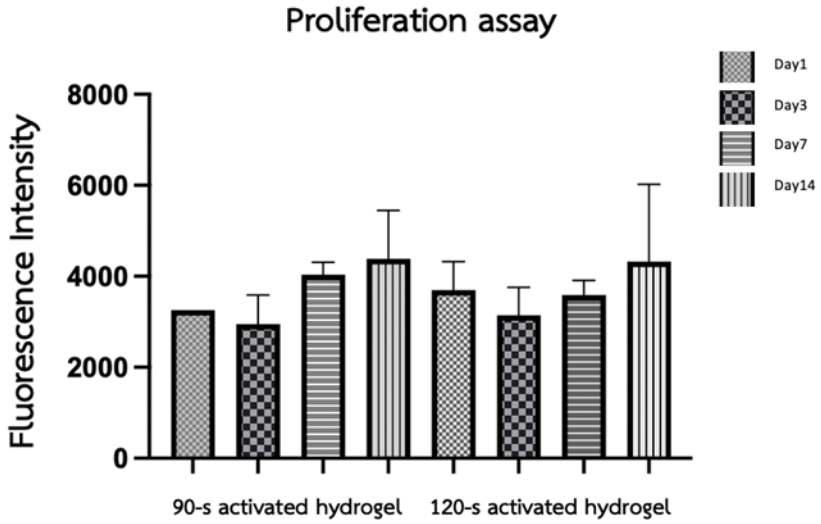

Figure 3 Bar graph of viability of cells, which was converted into the fluorescence intensity using resazurin assay.

DISCUSSION & CONCLUSION

HA hydrogel has been extensively researched as a biomaterial for tissue engineering. In a previous study by Chaopanitcharoen et al. (2022), MeHA hydrogel was developed using blue light to shorten the gelation time compared to the study by Areevijit et al (2021). This current study aimed to evaluate the biocompatibility of blue light activated MeHA hydrogel with mouse osteoblastic cell line, as a potential drug delivery system for bone tissue engineering.

The synthesized MeHA polymer demonstrated reproducibility with a degree of modification of approximately 40–60%. The pre-gel solution transformed into a gel in a similar time range to the previous study (Chaopanitcharoen et al., 2021), taking 90 to 120 seconds.

For hard tissue engineering purposes, mouse osteoblastic cells were cultured in the 3D model within the hydrogels, which provided more relevant conditions mimicking the in vivo cell behaviors. The study anticipated that MeHA hydrogels with different activation times would be biocompatible and have no negative effect on mouse osteoblastic cells. The proliferation assay showed a slow increase in metabolic rate from day 1 to day 14, with no statistically significant difference. However, the encapsulated cells remained viable at day 14 of culture. In contrast, Jivacharoen et al. (2022) evaluated the biocompatibility of blue light activated MeHA hydrogel with L929 fibroblast, which showed 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 strong enough to support cell proliferation (Rosaming et al., 2022). In agreement with the study by Zhang et al. (2017), primary osteoblast cultured in polydimethylsiloxane substrates with varying stiffness showed that the osteoblast spread widely with a polygonal shape on rigid substrate (~134 kPa) but were small and round when cultured on soft substrate (~1.4 kPa). Additionally, growth factors can be used due to their ability to regulate proliferation and differentiation of cells. Combining the hydrogels with signaling molecules such as growth factors could promote optimal tissue regeneration.

The current study demonstrated that MeHA hydrogels were biocompatible with 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.

In conclusion, blue light activated hyaluronic acid hydrogels were biocompatible with MC3T3-E1 mouse osteoblastic cell line. The cells were viable until day 14 of culture in 3D encapsulation. Therefore, this blue light activated HA hydrogel could be a promising drug delivery system for bone tissue engineering with an optimal gelation time. Further, this approach could be translated into in vivo model.

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Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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