Optimization of 3D Hyaluronic Acid-Alginate Hydrogel-based Culture System for Chondrocytes from Osteoarthritis Patient

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Abstract

Three-dimensional (3D) cell culture systems have been applied to several research designed to mimic the microenvironment of tissues, leading cells in these platforms to represent tissue more accurately than those grown in two-dimensional (2D) culture systems. Moreover, osteoarthritis (OA) is associated with excessive cartilage degradation, but the majority of pharmacological clinical studies were significantly effective under traditional 2D culture systems. Thus, these highlights represent the urgent requirement to enhance the understanding of the pathophysiology, therapeutic targets, and practical model development for OA drug selection. Therefore, this study aims to optimize the 3D cell culture conditions under non-scaffold-based and scaffold-based models on OA chondrocyte viability. The optimal model was evaluated using live/dead and morphological assay. The results of the non-scaffold-based culture system as a pellet culture model was optimized to the optimal condition of pellet size and rotation time under centrifugation using live/dead assay to 50,000 cell/pellet under centrifugation at 300x g for 5 min. In relation to the scaffold-based culture system as the hyaluronic acid -alginate (HA-Alg) hydrogel-based model, the hydrogel construction under the HA-Alg crosslinking on the construct size over the formulating obstructions was optimized to 250 µl. Moreover, cells under the HA-Alg hydrogel-based model were illustrated with the highest accuracy on cell viability and tissue mimetic morphology based on live/dead and morphological assay. In conclusion, the HA-Alg hydrogel-based 3D culturing conditions were optimized as a possible strategy for preserving the viability of OA chondrocytes and tendency development as an appropriate platform for the future application of OA drug screening.

Keywords**:** *3D cell culture systems, chondrocyte, hyaluronic acid -Alginate hydrogel, pellet culture*

1. Introduction

Osteoarthritis (OA) is a degenerative joint disease characterized by gradual cartilage destruction as well as inflammation of the surrounding synovial membrane, osteophyte production, and subchondral bone sclerosis. Moreover, it is caused by unclear multifactorial factors and the development of OA therapeutics are undergoing developments of OA therapeutics. Over this decade, OA treatments were classified into 4 majority groups including anti-degeneration, cartilage regeneration, bone resorption inhibitors, and anti-inflammatory therapies. However, a clinical review by Karsdal et al. (2016) revealed that most of the therapeutic candidates under *in vitro* studies were no substantial therapeutic effects. Thus, this highlight led to the urgent requirement for enhancing the understanding of the pathophysiology, therapeutic targets, and practical model development for OA drug selection. (Charlier et al., 2019; Karsdal et al., 2016; Yeung et al., 2019)

Three-dimensional (3D) cell culture systems have been applied to several research, especially studies related to therapeutic screening and pathophysiology in various diseases such as neurological disorders, cancer, and osteoarthritis. Underlying, these systems were designed to mimic the pathophysiological microenvironment of specific tissues influencing cellular behavior, bidirectional changes in cells and the extracellular matrix (ECM), concentration gradient on nutrients, oxygen, and waste, the dynamic variations regulating cell proliferation, differentiation, migration, survival, adhesion,

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cytoskeletal organization, and cell signaling. Therefore, these behavior changes led cells cultured in 3D platforms to better accurately represent tissue compared to those grown in two-dimensional (2D) culture systems as a planar monolayer on plastic surfaces. Especially, chondrocyte studies based on 2D culture systems during multiple passages found that chondrocytes underwent dedifferentiation as noticeable morphological and molecular changes. Thus, mimicking tissue conditions on cell culture is critical to understanding the disease phenotype and the development of more appropriate treatments. (Charlier et al., 2019; Langhans, 2018)

There are two fundamental types of 3D culture systems for chondrocytes including non-scaffold-based and scaffold-based systems. Regarding non-scaffold-based systems, the traditional model as well as pellet culture model-based spheres of a particular size are more adaptable to cellular and physiological gradients. However, the spheroid necessitated a large number of cells over the OA chondrocyte proliferation, leading to high throughput applications impractical. In another manner, scaffold-based models more accurately imitate cell-ECM interactions and were generally constructed from biomaterials such as hyaluronic acid (HA) and alginate (Alg), etc. In addition, scaffolds that provide physical support, are commonly utilized in investigations of primary chondrocytes to resolve issues of low cell proliferation and dedifferentiation.(Langhans, 2018; Yeung et al., 2019)

Hyaluronic acid (HA) is one of the most commonly used chondrogenic ECM components as a scaffold for 3D chondrocyte culture. Various studies have demonstrated that scaffolds containing HA can preserve many features of chondrocytes, including cell viability and dedifferentiated biomarker production at both the gene and protein levels, as determined by transcriptome analysis. (Kontturi et al., 2014; Ling et al., 2021) Moreover, there was evidence that incorporating HA into scaffolds can enhance stability and compatibility between chondrocytes and scaffolds, based on the presence of HA-specific receptors on chondrocyte membranes. (Hou et al., 2020) Consequently, these studies indicate that HA could be one of the potential components of the chondrogenic scaffold.

Although hyaluronic acid exhibits excellent chondrogenic biocompatibility and hydrophilicity, its application as a building block is limited due to its low viscosity, causing challenging gelation and low mechanical characteristics. Regarding the study by Thanh et al. (2022), the strategy for developing HA on the scaffold is compatible with a mechanically stable biopolymer as alginate (Alg) derived from brown algae, modified as a polymer network as a hyaluronic acid-alginate hydrogel and determined that this modified hydrogel can be used as a printable biomaterial in tissue engineering applications and promote cartilage tissue under mesenchymal stem cell study. (Thanh et al., 2022)

Therefore, this study aims to optimize the optimal formulating conditions for both the hyaluronic acid-alginate hydrogel-based model as the scaffold-based 3D culture system and the pellet model as the traditional 3D non-scaffold-based culture systems for the development of the HA-Alg hydrogel-based model to serve as the platform demonstrating the future potential for OA drug screening application.

2. Objective

To optimize the optimal formulating conditions on both the hyaluronic acid-alginate hydrogel-based model representing the scaffold-based 3D culture system and the pellet model representing the control on 3D culture systems

3. Materials and Methods

3.1 Ethical approval and OA cartilage sample collection

The study protocol was approved by the Human Ethics Committee of Thammasat University as MTU-EC-OT-4-233/62. In addition, each participant was provided with comprehensive information regarding the study's purpose and relevant issues. All patients submitted informed permission.

Articular cartilage samples were obtained from OA patients under diagnosed with Kallgren-Lawrence grade 3-4 radiographic OA and underwent knee replacement surgery at the Department of Orthopedic Surgery, Faculty of Medicine, Thammasat University Hospital, Thailand.

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3.2 Primary chondrocyte isolation and 2D monolayer culture

Primary chondrocytes were isolated modified protocol from paper described by Suntiparpluacha et al. (2016) under 20 hours of incubation with 0.2% collagenase type II (Sigma Aldrich, St. Louis, MO, USA) in HBSS (Gibco, Billings, MT, USA). Next, the isolated primary chondrocytes were cultured in culture media as DMEM/F12 containing 10% FBS and 1% Antibiotic-antimycotic (Gibco) in T25 and T75 flasks (NEST Biotechnology, Wuxi, China), incubated at 37 °C with 5% CO_2. Then, the growth of chondrocytes was monitored, and the cell culture medium was replaced every 2-3 days. Particularly, The primary chondrocytes were cultured using the 2D monolayer method until the second passage, at following passage they were trypsinized using 0.25% trypsin-EDTA (Gibco). (Suntiparpluacha et al., 2016)

3.3 3D pellet culture

The third passage of primary chondrocytes were cultured in 3D pellets as a control group applied from Yeung et al. (2016) protocol. Each pellet was generated by centrifugation of 50,000 and 100,000 chondrocytes in 500 µl of the culture media at 300 rcf for 5 and 10 minutes in partially uncovered screw cap microcentrifuge tubes (ExtraGene, Taichung, Taiwan). After 72 hours of incubation following figure 1, the pellets were transferred gently from the incubated tubes to a non-adhering 96-well plate as one pellet/well, containing 200 µl of culture media. Each well of 96-well plate (NEST Biotechnology) was coated with 2% agarose (SBIO by SmartScience, Pathum Thani, Thailand) in PBS (Gibco) as non-adhering property following Gadjanski et al. (2013) protocol. The growth of chondrogenic pellet was observed, and the cell culture medium was replaced every 2-3 days. Then, the optimal number of cells in chondrogenic pellet was selected using Live/dead assay during 7 days. (Gadjanski et al., 2013; Yeung et al., 2019)

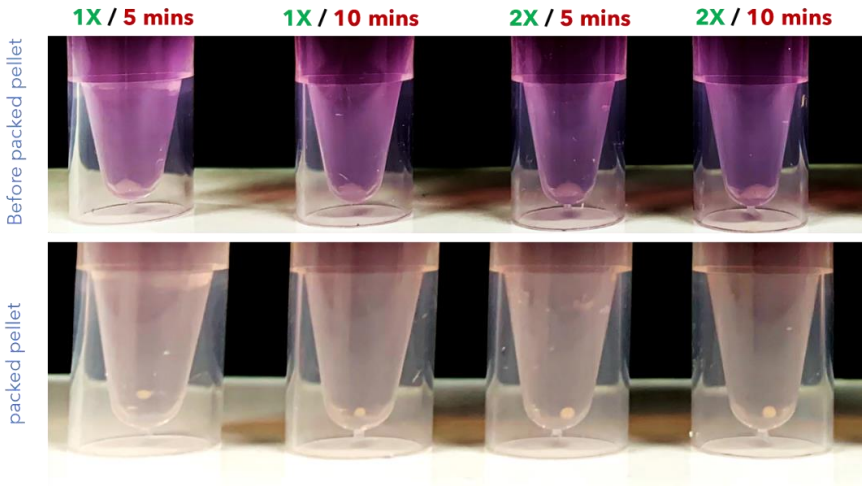

Figure 1 Packed OA chondrogenic pellet after 72 hours of incubation in partially uncovered screw cap microcentrifuge tubes: 1X and 2X were indicated 50,000 and 100,000 cells/pellet respectively under 300g centrifugation for 5 or 10 minutes.

3.4 HA-Alg hydrogel construction

HA-Alg hydrogel was constructed by mixing between 20 mg/ml modified aldehyde-alginate (Alg-CHO) and 30 mg/ml modified amine hyaluronic acid (HA-NH_2) as ratio 1:1 following the condition of Thanh et al. (2022) study. In this study, the constructs were molded in the various volume of mold as 50, 100, and 250 µl under 20 minutes gelation time. Next, the gelated constructs were observed under microscope for optimization the volume of mold over the formulating obstructions including bubble, wrinkle surface, formulating yield, following the ratio of actual construct number per theoretical construct number, and gel consumption. Then, the optimal volume of mold was selected. (Thanh et al., 2022)

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3.5 3D HA-Alg hydrogel based culture

The third passage of primary chondrocytes was encapsulated in a HA-Alg hydrogel construct using the protocol developed by Thanh et al. (2022) method. The primary chondrocytes as the selected optimal number of cells in chondrogenic pellet were dissolved with 500 µl of 20 mg/ml modified aldehyde-alginate. Next, the cell-Alg mixture was combined into 500 µl of 30 mg/ml modified amine hyaluronic acid and gently homogenized using spatula within 3 minutes. Next, the cell-Alg-HA mixture were transferred and divided into the selected mold using 1 ml syringe (Nipro Thailand corporation, Ltd., Phra Nakhon Si Ayutthaya, Thailand). After 20 minutes gelation, the galated cell-hydrogel constructs were transfer into a non-adhering 12-well plate as one cell-hydrogel construct/well, containing 2000 µl of culture media. Each well of 12-well plate (NEST Biotechnology) was coated with 2% agarose in PBS as non-adhering property following Gadjanski et al. (2013) protocol. (Gadjanski et al., 2013; Thanh et al., 2022)

3.6 Live/dead assay and morphology assay

The chondrocyte viability in 3D pellet culture and hydrogel-based culture were determined by Live/dead assay based on propidium iodide (PI) and Calcein-AM staining, identifying the dead cell as red fluorescence and the living cell as green fluorescence, respectively, modified protocol from paper described by Thanh et al. (2022). Especially, the pellets and HA-Alg hydrogel constructs were stained with 100 and 1500 µl of live/dead solution (Cellstain double staining kit, Sigma Aldrich, St Luis, MO, USA) and were incubated for 15 and 30 minutes at 37 °C with 5% CO_2, respectively. Then, the fluorescence of each living and dead signal and cell morphology were visualized under inverted fluorescent microscope. (Nikon ECLIPSE Ti–U, Nikon Corporation, NY, USA)

4. Results and Discussion

4.1 3D pellet culture

This study determined two independent criteria regarding chondrogenic pellet formulation, including the quantity of cells per pellet as 50,000 and 100,000 cells and the rotating time as 5 and 10 minutes. Thus, there were four pellet formulation conditions that were examined at three timepoints upon pellet transfer to an agarose-coated 96-well plate at the 0^{th} day, 3^{rd} day and 7^{th} day. Primarily, according to morphological visualization, the diameters of the OA chondrogenic pellets generated under all conditions gradually decreased from 690.83±106.06 µm at the 0^{th} day to 560.57±71.76 µm at the 3^{rd} day and 471.83±46.54 µm at the 7^{th} day according to the figure 2.

In parallel with the live/dead assay, the OA chondrogenic pellet under 10 minutes rotation revealed smaller diameter with higher condense packing than 10 minutes rotation both 50,000 and 100,000 cells size of pellets. Moreover, a higher number of dead cells regarding red fluorescence were approximately detected in the middle of the pellet. Then, these characteristics led to the determination that the OA chondrogenic pellet under 10-minute rotation was not selected as optimal conditions, as shown in figure 3. In addition, based on the low proliferation rate of OA chondrocytes, the OA chondrogenic pellet containing 50,000 cells, which exhibited the lowest red signal of dead cell on the 7^{th} day over 100,000 cells, was selected at the optimal formulation conditions with 5 minutes rotation.

In addition, based on the result the higher number of cells in pellet and longer rotation time with same centrifugal force cause the higher density of the pellet and tightly packed reducing cell viability in the middle area of the pellet under PI staining observation. Moreover, the results were consistent with the review article of Samvelyan et al., (2021) revealed that cells in the center of the pellet might be limited of nutrition and oxygen, necessitating the preservation of non-hypertrophic chondrocytes pellet. (Samvelyan et al., 2021)

However, the morphology of the cells within the OA chondrogenic pellet continued obscure and was not visible using this microscopy technique. To illustrate, following Yeung et al., (2019) report, H&E staining under histological technique was used as the effective method for observing the cells within OA chondrogenic pellet. (Yeung et al., 2019)

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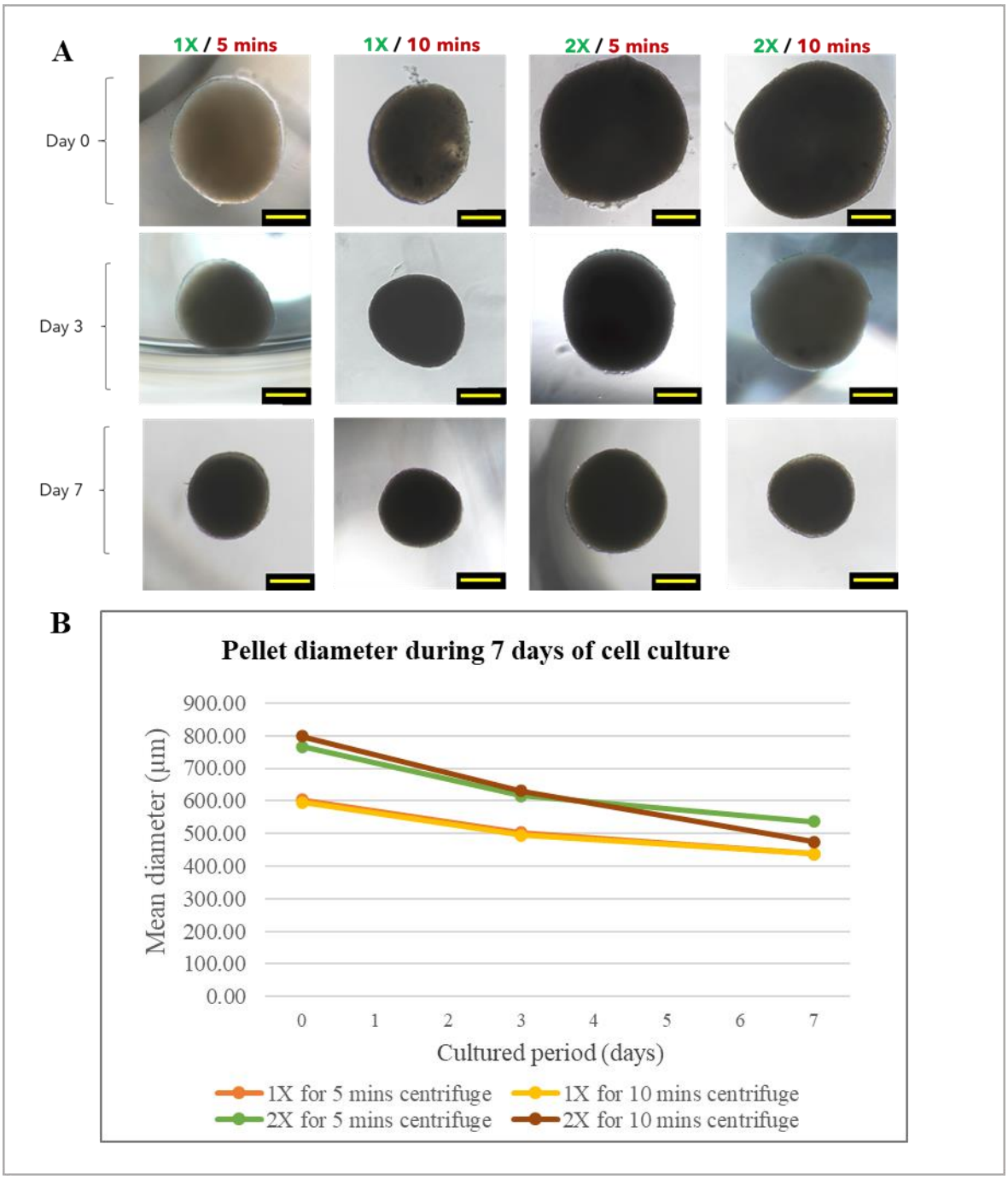

Figure 2 OA chondrogenic pellets during 7 days on OA chondrogenic pellets. In addition, (A) exhibited morphological visualization of pellets was observed under phase-contrast microscopy that there were 4 conditions of OA chondrogenic pellet formulation: 1X and 2X were indicated 50,000 and 100,000 cells/pellet respectively under 300g centrifugation for 5 or 10 minutes. (Scale bar = 200 µm) Meanwhile, (B) exhibited OA chondrogenic pellets diameter change during 7 days culture.

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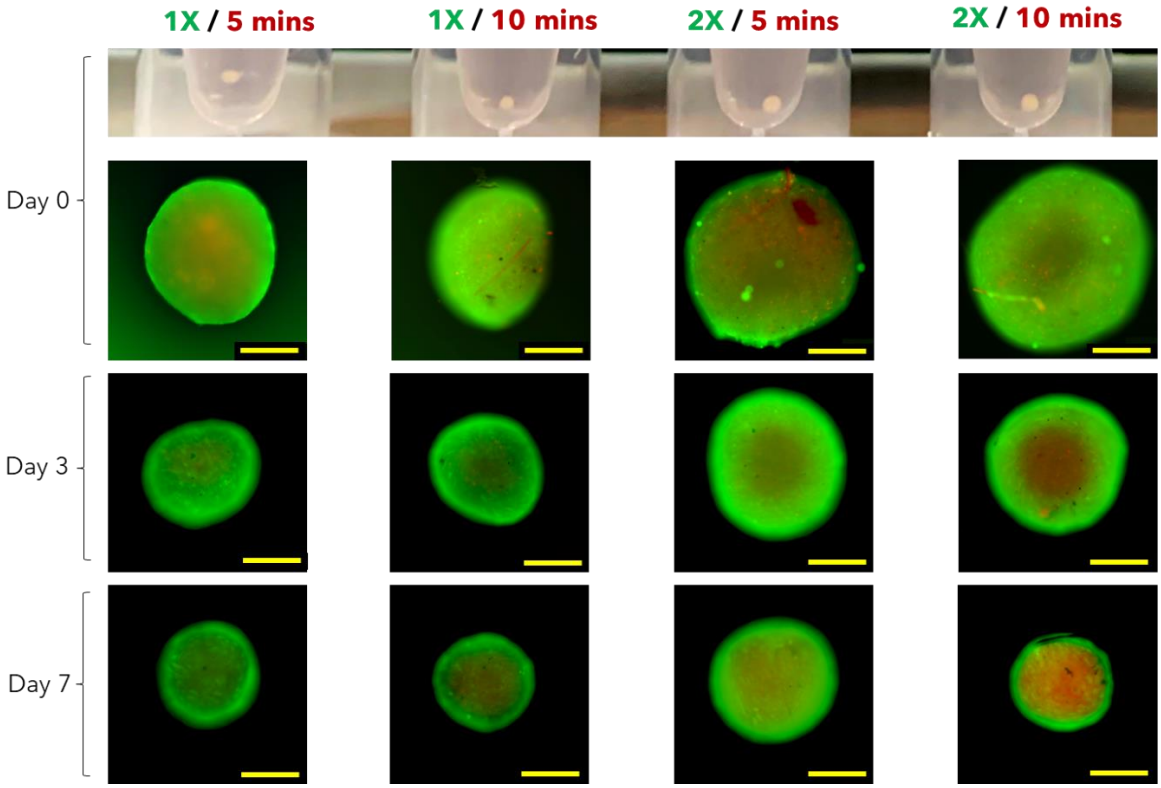

Figure 3 Live/dead visualization on OA chondrogenic pellets during 7 days under fluorescent microscopy. In addition, there were 4 conditions of OA chondrogenic pellet formulation including: 1X and 2X were indicated 50,000 and 100,000 cells/pellet respectively under 300g centrifugation for 5 or 10 minutes. (Scale bar = 200 µm)

4.2 HA-Alg hydrogel construction

According to HA-Alg hydrogel construction, the HA-Alg mixture were gelated within the three volumes of mold as 50, 100, and 250 µl under 20 minutes gelation time. As a result, the gelated constructs were evaluated under a microscope to optimize the volume of mold over the formulating obstructions including bubble, wrinkle surface, formulating yield, and gel consumption following table 1 and figure 4. In addition, the hydrogel constructs were ungently homogenized and were attached before complete HA-Alg crosslinking formation at 20 minutes, resulting in a HA-Alg hydrogel construct issue with a high number of bubbles and a surface with wrinkles, as shown in Figure 4A. Particularly, the 250 µl mold provided the hydrogel construct, experienced a lower bubble and wrinkle than the construct in Figure 4A having highest formulating yield. Consequently, the 250 µl mold was selected as the optimal volume of mold for HA-Alg hydrogel construction, highlighting on maximum formulating yield although consuming the largest gel volume.

Table 1 Observation on HA-Alg hydrogel construct using various molds.

Observation	250 µl-mold	100 µl-mold	50 µl-mold
bubble	no obvious difference	no obvious difference	no obvious difference
wrinkle surface	no obvious difference	no obvious difference	no obvious difference
formulating yield	1.00	0.75	0.50
gel consumption	high	medium	low

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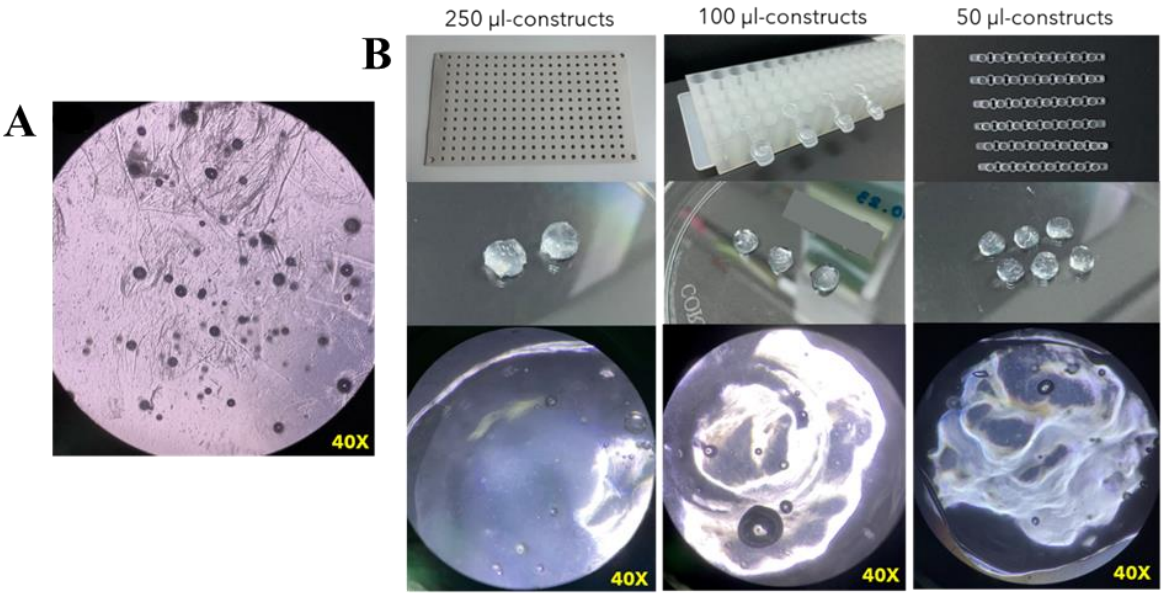

Figure 4 HA-Alg hydrogel construct visualization under 40X magnification. (A) exhibited the HA-Alg hydrogel construct issue with high number of bubble and wrinkle surface. (B) exhibited three various molds with their constructs and their microscopic observation.

4.3 3D HA-Alg hydrogel-based culture

According to 3D HA-Alg hydrogel-based culture, 50,000 OA chondrocytes were encapsulated with 250 µl of HA-Alg hydrogel per construct. Under 40X magnification, the live/dead displayed for the cell-hydrogel construct on the 7th day demonstrated no detection of PI signal, following in figure 5A. However, at a higher magnification of 200X, live/dead results on the cell-hydrogel construct at the 7th day were detected following figure 5B. This highlight can verify the potential of live/dead solution for detecting both of living and dead cell, despite the inability to detect the PI signal at low magnification due to the insignificant number of dead cells.

In addition, the live/dead visualization illustrated the cell patterns within this hydrogel-based culture higher cell-matrix interaction in comparison with the 3D pellet model representing more cell-cell interaction. Moreover, the result was consistent with Yeung et al., (2019) report, that their scaffold-based culture system as collagen microencapsulation on OA chondrocyte revealed a higher cell-matrix interaction than the non-scaffold-based culture system as 3D pellet culture. (Yeung et al., 2019)

Based on this result, the 3D HA-Alg hydrogel-based culture was verified as the appropriate platform for preserving osteoarthritis chondrocyte viability. The result was partially consistent with the study of Ling et al., (2021), in which both of their methacrylic anhydride (MA)-HA hydrogel-based culture and alginate hydrogel-based culture reported served the porcine primary chondrocyte viability that displayed high cell viability maintenance under live/dead assay, especially their MA-HA hydrogel constructs with high hydrophilic property mimicking *in vivo* environments for chondrocyte growth as indicated by their swelling assay. (Ling et al., 2021) Moreover, the report of Thanh et al. (2022), that encapsulated mesenchymal stem cells (hMSCs) within HA-Alg construct and then cultured them in chondrogenic medium, revealed hMSCs showed a homogeneous distribution in hydrogel consistent with the chondrocyte distribution in this study, and their live/dead results confirmed that hMSCs had high cell viability in hydrogel at the early stage of encapsulation within 7 day, with the potential to differentiate into chondrocytes. (Thanh et al., 2022) Therefore, both of the report showed the evidence that HA and alginate component in hydrogel can support the chondrocyte viability and development.

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In addition, the 3D HA-Alg hydrogel-based culture as the scaffold-based model demonstrated a higher viability of OA chondrocytes, particularly on the 7th day, and tended to be a more effective model than the typical 3D culture model without scaffold as pellet culture model. Consistently, the review article by Samvelyan et al., (2021) described that the biologic hydrogels derived from natural resources, containing a significant water component and their similarity to ECM modifiability, bioactivity, biodegradability, porosity, biocompatibility, and low immunogenicity, in addition to promoting chondrocyte viability and proliferation and collagen type II, aggrecan. Despite the report that cells in the middle of the pellet may lack gas and nutrients due to hypoxic and low nutrient conditions leading their viability is diminished. Consequently, the scaffold based model served the cell viability preservation more effectively than the traditional non-scaffold based model especially in long term culture. (Samvelyan et al., 2021)

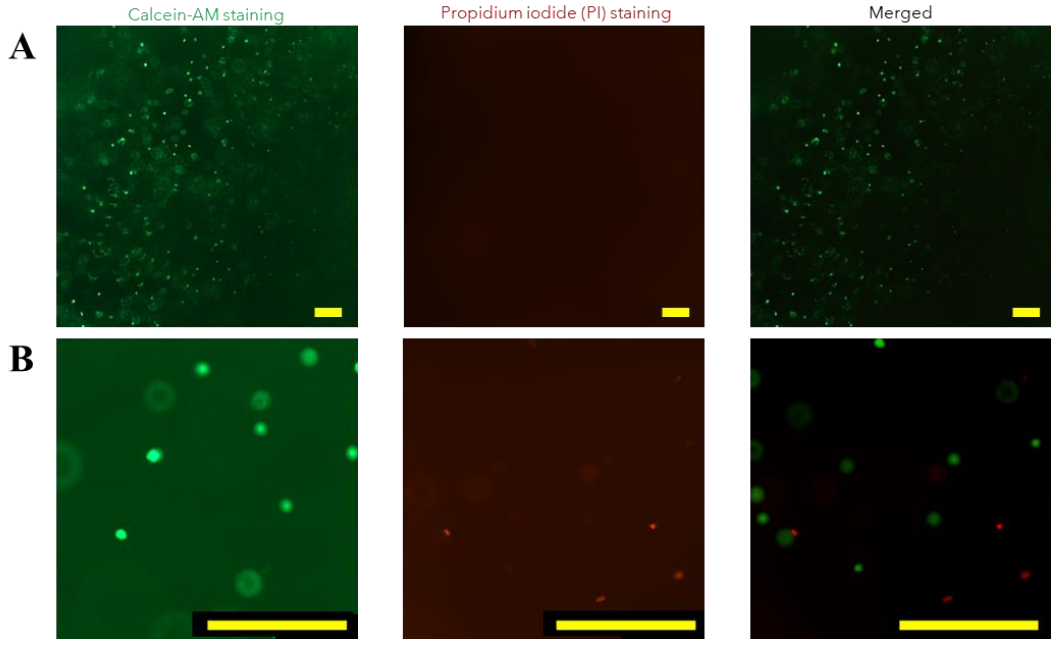

Figure 5 Live/dead visualization of cells in cell-hydrogel constructs at the 7th day. Particularly, Calcein-AM strained on the living cell fluorescing as green signal, whereas PI strained on the dead cell fluorescing as red signal. (A) and (B) exhibited cells in cell-hydrogel constructs under 40X and 200X magnification, respectively (Scale bar = 200 µm)

5. Conclusions

In conclusion, the HA-Alg hydrogel-based 3D culturing conditions were optimized as a possible strategy for preserving the viability of OA chondrocytes. In addition, the HA-Alg hydrogel-based model was validated as the platform for sustaining the viability of OA chondrocytes, demonstrating the future potential for OA drug screening application. However, the other timescale and other phenotype of OA chondrocytes in the HA-Alg hydrogel-based model, such as a potential OA and chondrogenic marker, should additionally be investigated for the preservation of the phonotype throughout the development of a culture model.

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