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# *In situ* hyaluronic acid-based hydrogel incorporated with insulin-like growth factor-1 for three-dimensional encapsulation of keratinocytes

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## ABSTRACT

**Background/Aim:** This work aimed to establish *in situ* hydrogel formation based on crosslinking of methacrylated hyaluronic acid (MeHA) and the polymer incorporated with insulin-like growth factor 1 (IGF-1) for keratinocyte three-dimension (3D) culture. **Materials and Methods:** The compatibility of human keratinocytes with developed hydrogel was determined by PrestoBlue™ cell viability assay. The live and dead cells were determined by Calcein AM, Propidium Iodide and Hoechst 33342 staining. The physical properties including gelation time, microstructure (porosity and interconnectivity), swelling ratio, and stability of the different concentration hydrogel were determined and correlated to cell viability. **Results:** Three percent w/v MeHA for *in situ* hydrogel formation resulted in the most suitable properties for the 3D cell culture. The 3% w/v MeHA hydrogel was used as a scaffold incorporated with various IGF-1 concentrations. The compatibility of the cells to hydrogel and effect of the growth factor were examined and found that the viability of the encapsulated cells was dramatically enhanced by incorporating IGF-1 into hydrogel in a dose-dependent manner. The established hydrogels incorporated with IGF-1 was established in this study and the hydrogels were demonstrated to be cytocompatible allowing the keratinocytes to grow in 3D fashion.

**Keywords:** Insulin-like growth factor 1, hyaluronic acid, keratinocytes, hydrogel, 3D cell culture

## INTRODUCTION

Hydrogels have been considered as biomaterials of choice for its biocompatibility with human tissues due to their hydrophilic property that is comparable to physical structure of extracellular matrix (ECM). Hydrogels can be fabricated to form the three-dimensional (3D) network with different synthesis techniques and processes of fabrication. Consequently, the hydrogel has been widely used in biology, clinics, drug delivery, and tissue engineering.[1] A two-dimensional cell culture is a conventional and simplified way of cell culture, which is commonly used to increase the number of cells for further experiments or to study basic interactions of the cells. However, the cells will grow and respond to the environment or stimuli virtually when they are cultured in the 3D environment that mimics real conditions of tissue. Therefore, hydrogel has become very popular scaffold for 3D cell culture and the powerful tool for monitoring cellular and external tissue formation.[2] The reactions between cell to cell and cell to matrix, which, herein, a cross-linked hydrogel network can be explored. Hydrogels possess suitable properties for the cell culture such as the high capacity to store water, physical properties that mimics the ECM and the solvent diffusion in the dispersion matrix.[2,3] The hydrogel has been widely applied as an effective structural model for 3D cell culture.[4,5]

Hydrogels are hydrophilic polymers that form 3D networks. The networks are mainly composed of the polymer chain with functionalities to initiate covalent bond or other bonds

between and/or within the chain polymers. When the long chains cross-link to each other with highly porous structure, the hydrogels were then fabricated with superior performance in water absorption.[6] The hydrogels are made from natural polymer and/or synthetic polymer. Researchers are able to make use of or design the polymers with the properties similar to that of ECM. It is considered very interesting for applications in tissue engineering. A lot of synthetic and natural materials may be used to make hydrogels for structural support in tissue engineering. Popular synthetic materials that have been used widely in tissue engineering include, for example, poly (ethylene oxide), poly (vinyl alcohol), poly (propylene fumarate-co-ethylene glycol (P (PF-co-EG)). Natural materials such as alginate, chitosan, collagen, and hyaluronic acid (HA) were also recognized as potential polymers in this application.[7] Natural polymer such as HA can be modified and used for the entire material fabrication. In other cases, they are used in combination with synthetic polymers such as poly (ethylene glycol) for certain purposes.[7-9] Hydrogel system for medical applications is designed to serve as a structural support for the cells that should be activated suitably for specific applications such as adherence, proliferation, differentiation, and tissue development without acute and chronic inflammatory responses. Hydrogels are typically designed to be non-toxic to delivering cells and surrounding tissues. HA is one of the most interesting component as the main structural element of the ECM and tissues in the body.[10,11] Theoretically, HA should be compatible with the body if they had not been contaminated during the experimental process.

In this paper, hydrogels can be formed based on the chemical modification of HA with methacrylic anhydride resulting in methacrylated HA (MeHA). The reaction between methacrylic anhydride and HA are the most common and widely used reaction for the HA modification. HA is a linear polysaccharide with repeating disaccharide subunit, composes of ß-1,4-D-glucuronic acid-ß-1,3-N-acetyl-D-glucosamine. There are many functional groups that are available for chemical modifications, for instance, carboxyl and hydroxyl groups. One of the most efficient reaction for fast crosslinking of HA chain to create polymer network of hydrogel can be thiol-ene Michael addition. Relying on such reaction, HA has to be modified with methacrylic anhydride creating the MeHA. Then, the gelation process can occur when the MeHA was bridged using dual-end-thiol-containing molecule. This reaction was first used by Grinstaff and team. Although this reaction did not give 100% yield, it was simple and efficient.[12,13] *In-situ* hydrogels are the form of hydrogel that can encapsulate cells simultaneously during the gelation process. Many studies have adopted *in situ* system in designing scaffolds for the cells encapsulation in 3D model. There are many advantages reported for the system. Cells encapsulation can create homogenous distribution of cell population throughout the material. Another win is the ability to create complex shapes/geometries as the gel solidify in the templates.[14,15] However, the gelation process of *in-situ* hydrogels may cause the toxicity to the cultured cell from chemicals or gelation processes such as ultraviolet light, heat, which may affect cell survival.[16,17] Thiol-ene Michael addition is a reaction that is convenient for the biological material preparation and is highly effective for the applications.[18] In this study, the gelation will used dithiothreitol (DTT) as a crosslinker. DTT is a linear thiol-tailored small-molecule that has been used in many reports as a crosslinking agent to create scaffolds for tissue engineering.[19] From previously reported work, HA-based hydrogels was used as a 3D scaffolds for study chondrogenesis of mesenchymal stem cells (MSCs) and bone defect repair.[20,21] Furthermore, it was also successfully applied in sealing of corneal abrasion and has wide range of applications in therapeutics.[22,23]

Insulin-like growth factor I (IGF-1) is a cytokine that is referred to the wound healing process. IGF-1 effects the growth similar to the action of insulin. Anabolic effects of IGF-1 are consisted of stimulation of DNA synthesis, cell proliferation, protein synthesis, and glucose transporters. During the wound healings, IGF-1 is increased significantly in wound fluid during the healing process, whereas, in normal skin layer, very low expression of IGF-1 is observed.[24,25] In normal skin, there are a few cells in the dermis and epidermis that could express IGF-1. However, all cells in the injured epidermis skin layer, macrophages and other cells can express IGF-1 for 1–3 days after injury. IGF-1 has a role to create the new blood vessels (angiogenesis), boosting collagen, as well as stimulation fibroblasts and keratinocytes. IGF-1 is mitogenic for keratinocytes and fibroblasts by inhibiting the apoptosis process. Consequently, it can reduce the cytokines production for inflammation and stimulates ECM production. Increasing of growth factors has been related with in the wound healing.[24-26]

In this case, we studied the suitable characterization of the hydrogels for keratinocyte cells culture in 3D condition for the incorporation of IGF-1. As IGF-1 is well established for application in wound healings, there is a high possibility that the platform reported here can bring about the development of materials and delivery system for wound treatment and skin regeneration. Moreover, the discovery could lead to further design for 3D model of skin tissue as *in vitro* second skin platform for activity and toxicity assessment of active pharmaceutical compounds.

## MATERIALS AND METHODS

### Cells and Reagents

HA (Molecular weight = 403.1 g/mol) was generous gift from Changchun Institute of Applied Chemistry. Methacrylic acid (Molecular weight = 154.16 g/mol) was purchased from Sigma Chemical, Inc. (St. Louis, MO, USA). DTT (Molecular weight of DTT = 153.253 g/mol) was purchased from Sigma Chemical, Inc. (St. Louis, MO, USA). Immortalized human keratinocyte (HaCaT) cell line were purchased from Cell Lines Service (Heidelberg, Germany) and used between passage 12 and 18. HaCaT were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum, 2 mM L-glutamine and 100 units/ml of each penicillin and streptomycin (Gibco, MD, USA) at 37 °C in a 5% $CO_2$ atmosphere. IGF-1 was purchased from Sigma (St. Louis, MO, USA). Resazurin-based reagent (PrestoBlue™) was from Invitrogen (Carlsbad, CA, USA). Hoechst 33342, Calcein AM and propidium iodide (PI) were obtained from Molecular Probes Inc. (Eugene, OR, USA)

## Polymer Synthesis

*MeHA*

MeHA was synthesized using 1% w/v of 47 kDa HA. HA was dissolved in the potassium phosphate buffer at pH 8. Methacrylic anhydride was gradually added in 10 folds of HA monomer into the HA solution at 0°C, the pH of 8 was maintained using 5M NaOH solution and continued the reaction for 24 h at a temperature of 4°C throughout the reaction. Then, the reaction was brought to dialysis at 4°C at least for 48 h. The reaction solution was centrifuged to eliminate precipitate out. The product was frozen at a temperature of −20°C and was lyophilized until dry. The MeHA polymer was analyzed with $^1$H NMR 300 MHz to find out the degree of modification. That were calculated as the following equation.[20]

$$\text{Degree of modification} = (H_{Me}/H_{HA}) \times 100$$

$H_{Me}$ = Integration of H peak at methyl group of methacrylic anhydride
$H_{HA}$ = Integration of H peak at methyl group of hyaluronic acids

*Study of gelation time*

Preparation the MeHA polymers by dissolving 3% and 5% w/v MeHA in the phosphate buffer saline solution (PBS) 500 µl, adjust to pH 8 with 5M NaOH. Adding the dithiotreitol (DTT) as a cross-linking agent with the concentration of 0.1M in the ratio of thiol: ene = 1: 2. Measuring the gelation time were used a vial tilting method.[27] The sample was observed no flow when inverted each eppendorf for 20 s.

*Study the swelling property of the hydrogel*

The MeHA hydrogel 250 $\mu$l with the different concentrations of MeHA was immersed in PBS at room temperature for 2 days. The hydrogels was weighed over times. The excess PBS was blotted out from the hydrogel with Whatman filter paper before weighing. The weight of swollen hydrogel ($W_s$) obtained from the hydrogel after swelling and the dry mass ($W_d$) obtained from these hydrogels after lyophilization. The mass-based swelling ratio ($S_m$), the volumetric-based swelling ratio ($S_v$), and water content were calculated as the following.[28]

$$\text{The mass-based swelling ratio } (S_m);\ S_m = W_s/W_d$$

$S_m$ = The mass-based swelling ratio
$W_s$ = Weight of hydrogel after swelling
$W_d$ = Weight of hydrogel after lyophilization
The volumetric-based swelling ratio ($S_v$); $S_v = 1 + \rho_d/\rho_s\ (S_m\text{-}1)$
$S_v$ = The volumetric-based swelling ratio
$\rho_d$ = The density of the dry MeHA polymer (1.229 g/cm$^3$)
$\rho_s$ = The density of the solvent (1 g/cm$^3$ for PBS)
The percentage of water content; Water content = $(W_s - W_d/W_s) \times 100$

*Analysis of stability of hydrogel*

Hydrogels 250 µl were immersed in PBS 1 ml at room temperature, then recorded the reduction of hydrogel weight each time point. The hydrogels were collected and lead to lyophilize for at least 24 h before weighing.[20] The initial dry weight was determined as $\mu_0$. The dry weight at each time point was determined as $\mu_t$. The ratio of weight loss of hydrogels was calculated as the following equation, $\mu_t/\mu_0$.

*Study the microstructure*

Hydrogel structure with the concentration for gelation of the polymer MeHA equal to 3% and 5% w/v. Analysis the pore size and pore distribution using a Scanning Electron Microscope (SEM). The dry hydrogels after lyophilization were cross-sectioned and attached to the stub with carbon tape. The samples were coated with gold before analyzing, random samples for the 3–5 positions. Measuring the pore diameter showed result as the mean ± standard deviation.

*Cell viability assay of MeHA with different concentrations*

Analysis the cell viability using PrestoBlue™ assay, measured after day 3, 5, 7, and 10 of the keratinocytes culture in the hydrogel. Briefly, $1 \times 10^5$ cells/ml of HaCaT was encapsulated in 96 well plates with the 3% and 5% w/v MeHA concentration. The DMEM media in each well was replaced by DMEM 100 ml with 10% PrestoBlue™ and incubated for 30 min at 37°C and 5% $CO_2$. The DMEM media containing 10% PrestoBlue™ without cell was used as negative control. Measuring the fluorescence intensity at 560/590 nm (Ex/Em) using a microplate reader (Anthros, Durham, NC, USA).

*Cell viability assay of IGF-1 with different concentrations in the appropriate hydrogels*

Analysis the cell viability using PrestoBlue™ assay, measured after day 3, 5, 7, and 10 of the keratinocytes culture in the hydrogel from previous experiments with various concentration of IGF-1 (10, 50 and 100 ng/ml). The DMEM media in each well was replaced by DMEM 100 ml with 10% PrestoBlue™ and incubated for 30 min at 37°C and 5% $CO_2$. The DMEM media containing 10% PrestoBlue™ without cell was used as negative control. Measuring the fluorescence intensity at 560/590 nm (Ex/Em) using a microplate reader (Anthros, Durham, NC, USA).

*Study of Live/Dead Cell Viability*

The HaCaT Cells were cultured on 96 well plate ($10^5$ cells/ml in each well) with hydrogels incorporated various IGF-1 concentration (10, 50, and 100 ng/ml). After day 3, 5, 7, and 10 washed with PBS for 3 times. The live cells and dead cells have been analyzed with Calcein-AM and PI, respectively. The cells were fixed with the 4% paraformaldehyde solution at room temperature for 15 min and analyzed the cell nucleus with Hoechst 33342 100 ng/ml. Using a fluorescence microscope (Axio Observer Z1, ZEISS, Germany) at 495/515 nm (Ex/Em) to observed the live/dead keratinocyte cells.

## Statistical Analysis

All quantitative data results were presented as mean ± standard deviation (SD). Statistical analysis is performed using T-test and One-way ANOVA, with a significance level of 0.05 ($P < 0.05$).

# RESULTS

## Polymer Synthesis

*MeHA*

The resulted lyophilized MeHA was fibrous and bulky. MeHA polymers with degree of modification in the range of 50-80%

could be defined by $^{1}$H NMR [Figure 1]. The resulting MeHA could be dissolved in the designated concentration and cross-linked by DTT to successfully create the hydrogel through Thiol-ene reaction.

*The effect of polymer concentrations on physical properties of MeHA hydrogels*

MeHA polymer was dissolved in PBS with concentration of 3% and 5% w/v of MeHA, adjust to pH 8 with 5M NaOH. DTT solution with concentration of 0.1M as a cross-linking agent were added with a mole ratio of thiol: ene = 1: 2. The gelation time was observed from the transition of solution-gel phase. The solution-gel phase transition of the 5% MeHA hydrogels appeared earlier than the 3% MeHA hydrogels. The average gelation time of the 3% and 5% w/v MeHA hydrogels were 25 and 20 min, respectively [Figure 2a]. We observed that the gelation time was correlated favorable to the polymer concentrations of the hydrogels therefore the hydrogels at high polymer concentration indicated significantly faster gelation time than the hydrogels at low polymer concentration.

The microstructure was investigated by SEM as shown in Figure 2b. Both 3% w/v and 5% w/v MeHA hydrogels revealed a similar pore shape and pore size. Both hydrogels approximately appeared 10-100 μm range of pore size. The 3% w/v MeHA hydrogels showed high porosity and rougher surface. In addition, the cross-section also demonstrated well interconnectivity with thin layer of outer edge. The 5% w/v MeHA hydrogels presented lower porosity and interconnectivity with thick layer of outer edge. Furthermore, we observed the much denser dry mass of polymers from the 5% MeHA hydrogels comparing to the 3% MeHA hydrogels with less porosity. The outer surface of 5% MeHA hydrogels was also denser and smoother.

The swelling ratio of both MeHA hydrogels reached the equilibrium after soaked in PBS for 2 days. The gel weight ratios, swelling ratios, and water content of both MeHA hydrogels are shown in Figure 3. Both MeHA hydrogels could absorb water quickly in the early 8 h and continued with slight increas at later time [Figure 3a]. Both hydrogels had water content more than 95%. The water content and swelling ratio ($S_m$ and $S_v$) of the 3% w/v MeHA hydrogel was significantly higher than the 5% w/v MeHA hydrogel.

The stability of the hydrogels was observed by the weight loss after being immersed in PBS for 11 days. The results showed that the weight loss was not found in both MeHA hydrogels over experimental period [Figure 3e].

*The effect of polymer concentrations on keratinocyte cell viability of MeHA hydrogels*

PrestoBlue™ assays were used in the HaCaT cell viability assays of the MeHA hydrogels. The HaCaT cells were encapsulated in both hydrogels and cultured over 10 days. The fluorescence intensity of PrestoBlue™ product resulting from metabolism of the viable HaCaT cells are shown in Figure 4. Therefore, the numbers of viable cells could be reflected from differences in the fluorescent intensity. The fluorescence intensity detected from the culture in the 3% MeHA hydrogel was significantly higher than the 5% MeHA hydrogel in all time points, excepted at day 5 [Figure 4a]. The fluorescence intensity of the 5% MeHA hydrogels was significantly increased from day 5 to day 7. Both hydrogels showed the fluorescent intensity steadily increased from day 3 to day 7. After that, the reduction of fluorescence intensity was observed at day 10. However, the fluorescence intensity of the 3% MeHA hydrogels at day 7 demonstrated significantly higher than the 5% MeHA hydrogels. From this data, the 3% MeHA hydrogel was chosen

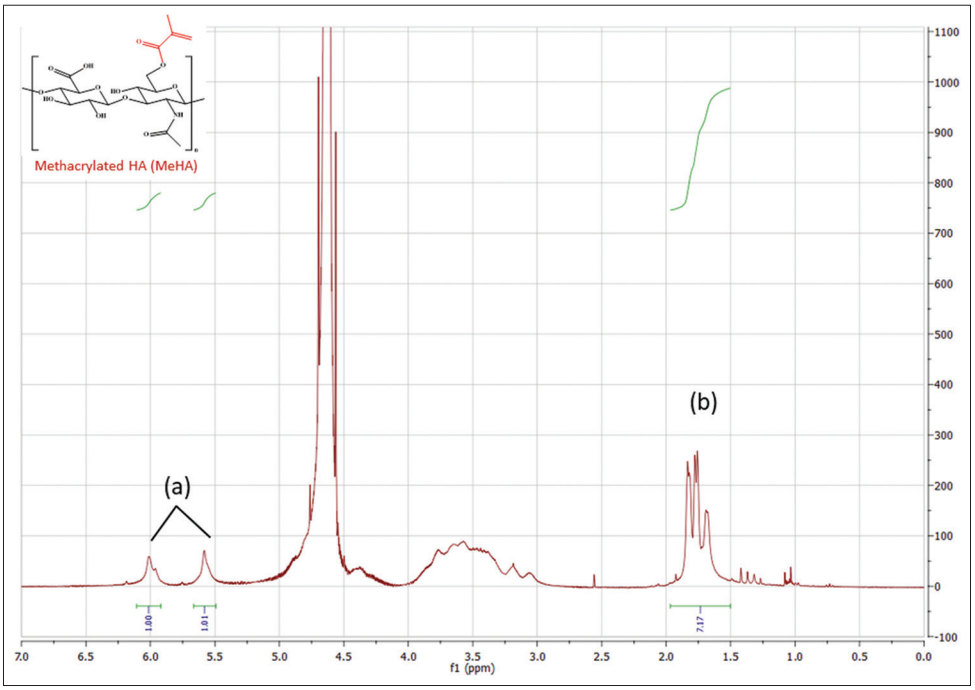


**Figure 1:** $^{1}$H NMR spectra of the MeHA polymer with the degree of modification was approximately 70%. $^{1}$H NMR peaks at the vinyl group of methacrylate represented by (a). $^{1}$H NMR peaks for overlapping methyl groups on the methacrylate and hyaluronic acids represented by (b)

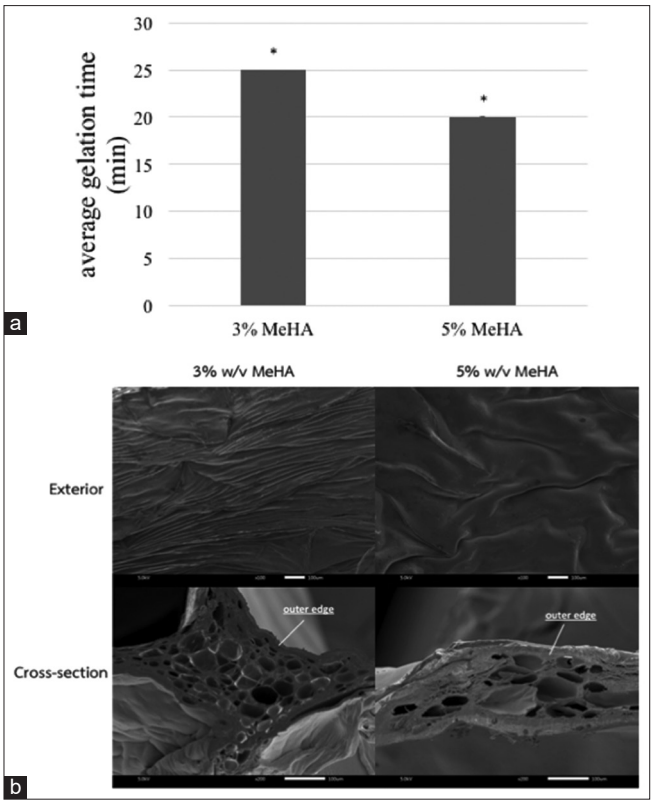


**Figure 2:** (a) The average gelation time of the 3% MeHA and 5% MeHA hydrogels. The values displayed are means ± SD, $n = 3$. Statistics: *independent sample *t*-test, $P < 0.05$. (b) Surface and cross-section SEM micrographs of the MeHA hydrogels after freeze-drying. Scale bar is 100 μm

as the more suitable for 3D keratinocyte cell culture to be used for later experiments.

*The effect of IGF-1 concentrations on keratinocyte cell viability of 3% w/v MeHA hydrogels*

The encapsulated HaCaT in the 3% w/v MeHA hydrogels at different concentration of IGF-1 (10 ng/ml, 50 ng/ml and 100 ng/ml) was examined by the PrestoBlue™ cell viability assay [Figure 4b]. The addition IGF-1 did not significantly affect gelation of our hydrogel system. Only with 100 ng/ml IGF-1 formulation, we observed slightly slower gelation time while incorporating cells, *in situ*. The metabolic activity of viable HaCaT cells in the MeHA hydrogels without IGF-1 was significantly increased from day 3 to day 7 and significantly decreased at day 10. In contrast, the MeHA hydrogels with various IGF-1 concentrations was continuously increased from day 3 to day 10 without any drops. The MeHA hydrogels with 100 ng/ml IGF-1 showed the highest fluorescence intensity with statistically significant at day 10. The fluorescence intensity from day 7 to day 10 were greatly increased from cell culture within MeHA hydrogels with 100 ng/ml IGF-1, following by 50 ng/ml and 10 ng/ml IGF-1, respectively. However, the overall trend of increased metabolic rate revealed that the addition of IGF-1 at concentration of 100 ng/ml can effectively enhance metabolic rate in all time points. Therefore, IGF-1 could further promote cell viability of the MeHA hydrogels such that IGF-1 with higher concentration showed higher viable cells.

The viable cells were evidenced by the fluorescence images [Figure 4c]. Calcein-AM (CAM), PI and Hoechst 33342 displayed viable cells, dead cells and nucleus, respectively. We observed that the viable cells at day 7 were increased according to the IGF-1 concentrations by observing the green color of the fluorescent images. This is in correlation with the results from PrestoBlue™ assay. The fluorescence images showed the more intense staining with higher amount of viable cells in the MeHA hydrogels with higher IGF-1 concentrations. However, due to the feature of 3D hydrogel culture, HaCaT cells was not localized on one plane of the 3D scaffolds resulting in difficulty in acquiring clear fluorescent images. The fluorescent images could eventually support that the HaCaT cells could survive and compatible with *in situ* fabrication of our proprietary MeHA hydrogel.

## DISCUSSION

This study described the concentration effect of MeHA polymers on physical properties of the proprietary *in situ* HA hydrogel and keratinocyte cell viability when encapsulated 3D. The biological activity of IGF-1 incorporated in the hydrogel was further explored with different concentrations on keratinocyte cell viability with 3D culture.

In this study, we used 3% and 5% w/v MeHA polymer concentration, DTT was used as a crosslinking agent for the gelation. The gelation reaction was the Michael addition reaction (Thiol-ene reaction),[20] which occurred between the thiol group of DTT and the alkene groups on MeHA polymer. The molar ratio of thiol: ene was kept constant at 1:2. Gelation time decreased as the concentration of MeHA polymer increased from the more readily available functional groups for crosslinking under the controlled condition.[27,29,30]

After gelation, the hydrogel underwent lyophilization for the observation of porosity and pore size of the hydrogel using SEM. It showed that 3% w/v MeHA hydrogel had more porosity and pore interconnectivity. The swelling study of hydrogel revealed that water content and swelling ratio of 3% w/v MeHA hydrogel increased significantly comparing with 5% w/v MeHA hydrogel. The swelling ratio was related to the porosity and pore interconnectivity of the hydrogels. The swelling ratio was increased according to the higher porosity and well interconnectivity. Sannino *et al*., studied on swelling kinetics of porous and nonporous hydrogel. The showed that swelling process of the porous hydrogel was faster than nonporous hydrogel.[31] The explanation focused that the water molecule can be allocated and penetrated better within the material with high total pore volume. We supposed that the necessary nutrients soluble in cell culture media critical for cell growth could be permeated into the hydrogel and help to support for the cell growth.

Hydrogels have been used as scaffolds for the 3D culturing of keratinocyte cells. Therefore, cell viability would be the first thing we considered for 3D cell culture in the hydrogel. The compatibility of hydrogels towards cells was measured from cell viability via metabolic activity and live/dead staining. PrestoBlue™ cell viability assay was used to measure the metabolic activity of viable keratinocyte cell for the different polymer concentration and different days of culture. The

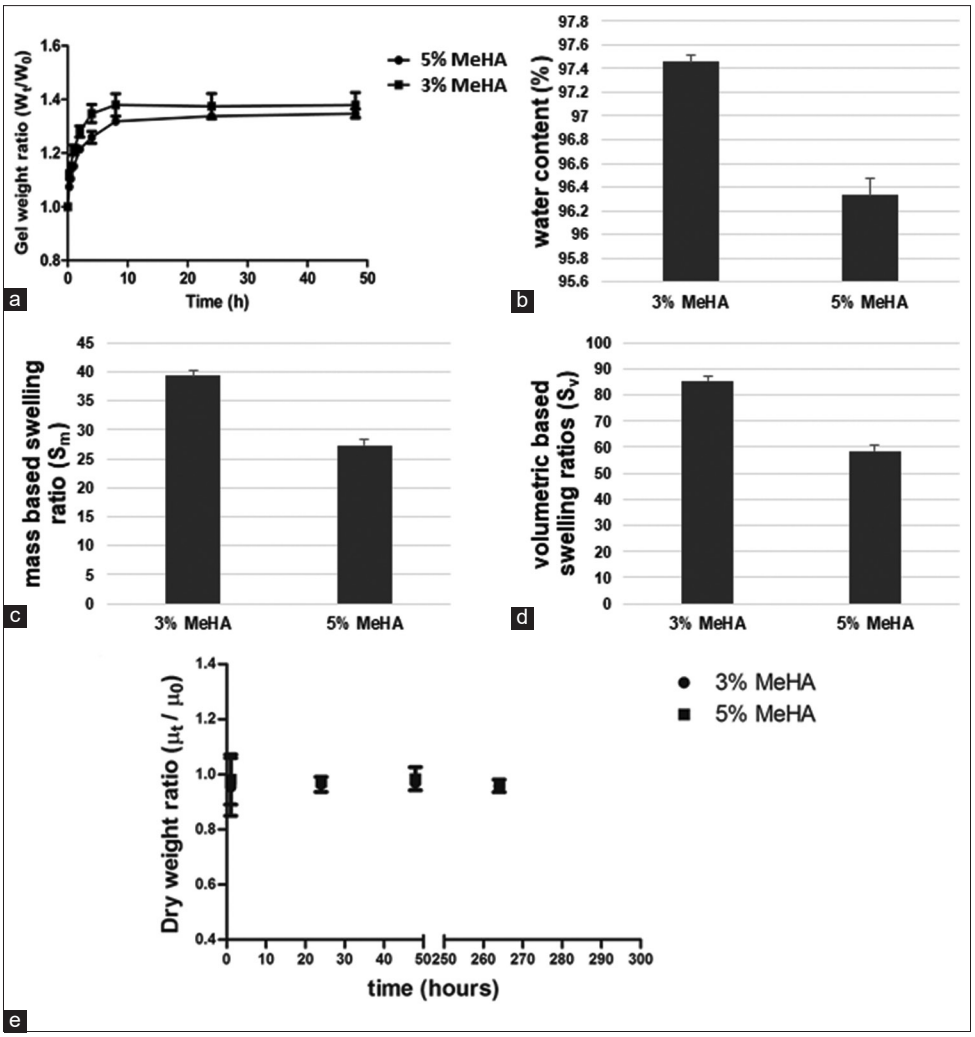


**Figure 3:** (a) Hydrogel weight ratio. (b) Water content. (c) Mass-based swelling ratio. (d) Volumetric-based swelling ratio of the MeHA hydrogels after soaked in PBS for 2 day. (e) The stability of the MeHA hydrogels. The weight loss was monitored for 11 days. The values displayed are means ± SD, $n = 3$. Statistics: independent sample *t*-test, $P < 0.05$

cells showed continuous increase in metabolic activity from the starting point to day 7. However, the detectable cellular activity dropped on day 10. The possible explanation could be that (1) the cells were over confluent due to the high density of cells in the hydrogel matrix, (2) the cells could change their behavior leading to preserved metabolic activity. The second explanation involved cell differentiation, as Seo, *et. al*., 2005 suggested that metabolic activity of keratinocytes is generally decreased during the differentiation process, coinciding with the decrease in cell proliferation.[32] Another interesting point from comparing the encapsulated cell viability in different concentrations of polymer-forming *in situ* gel was observed. The viscosity of the polymer solution before gelation (pre-crosslinked) was important for maintaining cell viability during the *in situ* cell encapsulation process. The viscosity could be altered in many ways, for example, by the molecular weight of polymer and by the polymer concentration before the gelation. Furthermore, the viscosity of polymer solution could consequently affect the hydrogel network crosslinking density. In our case, the viscosity of the polymer solution can be caused by the higher polymer concentration.[33,34] The high viscosity pre-gel polymer solution resulted in high shear modulus when mixing the cell,[35] that could potentially destroy cell membranes and hindered the cell-cell interaction. As a result, the cell viability was decreased.[36,37] For above-mentioned reasons, we chose hydrogel formulation with lower polymer solution concentration (3% MeHA) to incorporate IGF-1 for further the study.

The IGF-1 concentrations involved in the viability of cells, due to the reported biological function of IGF-1 in supporting cell proliferation in many 2D culture models.[38,39] Herein, the function of IGF-1 under 3D keratinocyte cell culture was compared between the MeHA hydrogel with and without IGF-1. We observed that the MeHA hydrogel with IGF-1 could further facilitate the proliferative keratinocytes from day 7 to day 10 in 3D manner, while the MeHA hydrogel without IGF-1 decreased the metabolic activity of viable cells on day 10. [Figure 4a]. Fluorescence images obtained from fluorescence microscope, could supported the result from cell viability assay. We found that more amounts of viable cells could be measured following the concentration of IGF-1. The viable cells were observed by the specific florescent staining with Calcein AM, giving out

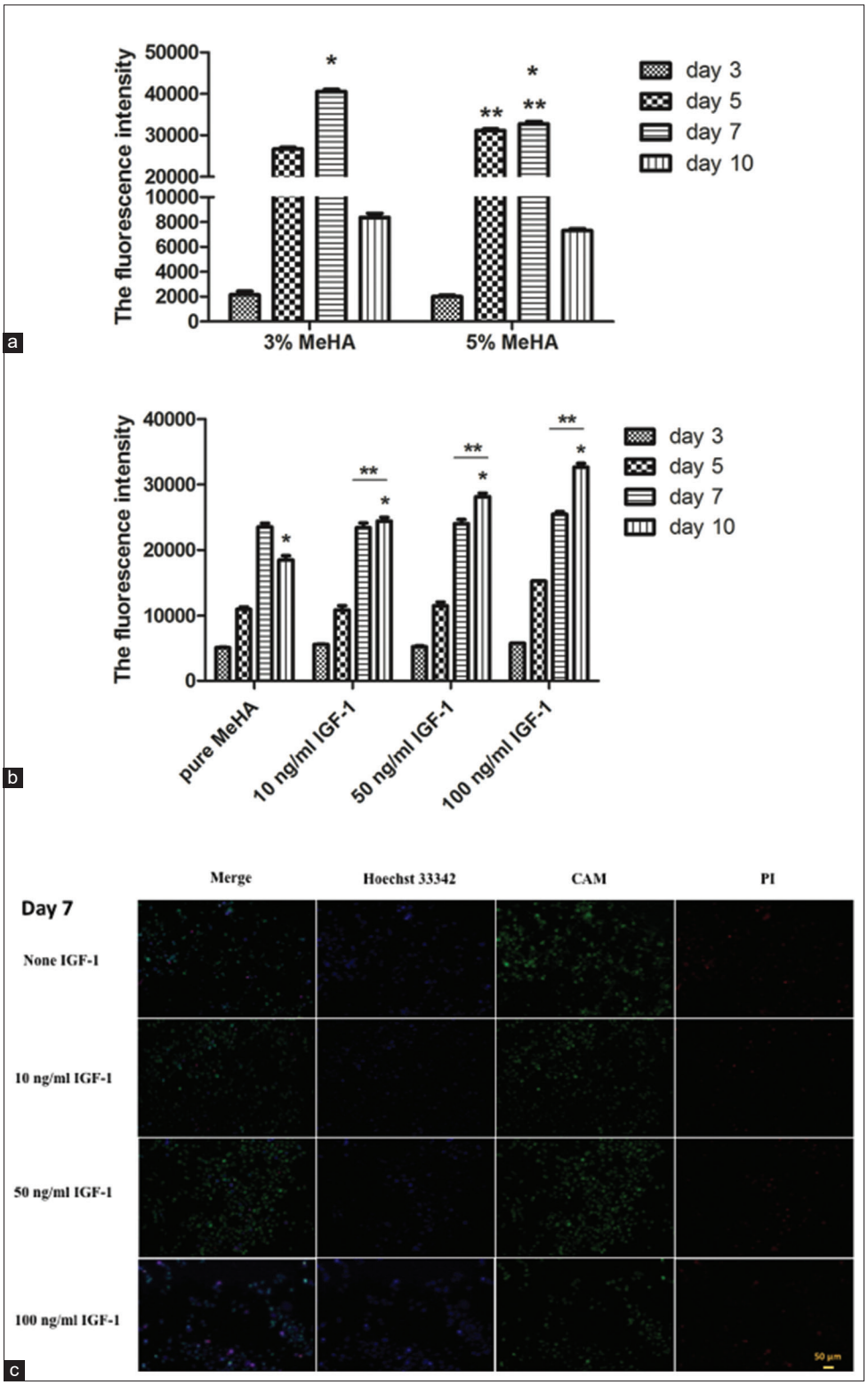


**Figure 4:** (a) The fluorescence intensity from metabolic activity of viable HaCaT cells encapsulated in the MeHA hydrogels detected by PrestoBlue™ assay. The values expressed are means ± SD, $n = 3$. Statistics: *independent sample *t*-test, $P < 0.05$, **paired-sample *t*-test, $P < 0.05$. (b) The fluorescence intensity reflecting from metabolic activity of viable HaCaT cells encapsulated in the 3% w/v MeHA hydrogels at different concentration of IGF-1, detected by PrestoBlue™ assay. The values expressed are means ± SD, $n = 3$. Statistics: *one-way ANOVA with independent sample *t*-test, $P < 0.05$ (compared each IGF-1 concentrations at day 10), **paired-sample *t*-test, $P < 0.05$ (compared each IGF-1 concentration at day 7 and day 10). (c) Fluorescence images of HaCaT cells encapsulated in the 3% w/v MeHA hydrogels at different concentration of IGF-1. The scale bars for all images is 50 μm

green fluorescence at 495/515 nm (ex/em) wavelengths. The metabolic activity of the cells encapsulated within the hydrogel with IGF-1 at 100 ng/ml was the highest among other concentrations with statistically significant on day 10. This result was supported by many researches presenting that IGF-1 can accelerate wound healing. IGF-1 implicate the stimulation of wound healing by increasing keratinocytes migration[40] and proliferation. Ingo, *et. al.* discovered substantial function of IGF-1 that stimulated plasma membrane protrusion. The spreading in epidermal keratinocytes led to accelerate the wound epithelialization.[41] Taken all together, we expected that this hydrogel platform will be useful for further research and development of materials for wound healings.

## CONCLUSION

In conclusion, our result demonstrated that 3% w/v MeHA hydrogels was considered an appropriate scaffold for the 3D keratinocyte cell culture. The concentration of MeHA polymer affected the physical properties of hydrogels such as gelation time, swelling ratio, microstructure (pore size, porosity and pore interconnectivity), and cell viability. Increasing the concentration of MeHA polymer led to faster gelation time, decreased swelling ratio, porosity, pore interconnectivity and eventually decreased cell viability. Therefore, the 3% w/v MeHA hydrogels was used to continue the test for growth factor effect for 3D keratinocyte cell cultured with various concentrations of IGF-1. As result, the MeHA hydrogels with 100 ng/ml IGF-1 increased cell viability and proliferation of keratinocyte cells higher than other IGF-1 concentrations. For the future work, this MeHA hydrogel incorporated with IGF-1 could be applied and further developed as scaffold for research involving wound healings and skin regeneration.

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## CONFLICT OF INTEREST

The authors declare no potential conflicts of interest.

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