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INFLUENCE OF PHOTO-INITIATOR CONCENTRATION ON THE VIABILITY OF CELLS ENCAPSULATED IN PHOTO-CROSSLINKED MICROGELS FABRICATED BY MICROFLUIDICS

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Photo-initiators have long been used as a simple and effective photo-polymerization tool for biomedical applications such as cell-embedded scaffolds. Nevertheless, the influence of photo-initiators on cytotoxicity has not yet been comprehensively studied. Thus, we investigated the viability of cells encapsulated in gelatin methacrylate (GelMa) microgels in the presence of different concentrations of photo-initiator. GelMa microdroplets (5wt%)containing different concentrations of photo-initiator (0.2, 0.5, 0.7, and 1.0 wt%) were generated using a flow-focusing microfluidic device. Cells grown in GelMa microgels containing 0.2 wt% photo-initiator had high viability, suggesting that the photo-initiator was not cytotoxic at this concentration. However, at photo-initiator concentrations greater than 0.5 wt%, cell viability began to decrease; moreover, at 1.0 wt%, almost no live cells were observed. The decreased cell viability associated with increased concentrations of photo-initiator is probably a result of residues and byproducts from reactions with photo-initiators. This detailed study of the relationship between cell viability and photo-initiator concentration can inform the development of future photo-crosslinking strategies used in cell-laden hydrogel applications.

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1. Introduction

Biomaterials used in biomedical applications, such as regenerative medicine and drug delivery, have often been designed to form gels by photo-initiated polymerization [1, 2]. The possible applications of photo-polymerized hydrogels include acting as a barrier to prevent tissue injury, providing a drug delivery system for in vitro targets, and forming a scaffold for cell growth and transplantation [3-5]. For example, a polyvinyl alcohol (PVA) hydrogel has been photo-crosslinked and used in combination with a cell-adhesive peptide to support cell attachment and spreading [6]. A biodegradable scaffold based on photo-polymerizable poly(lactic acid)-g-PVA hydrogel has also been utilized to fabricate engineered heart valves [7]. In addition, photo-polymerizable poly-(ethylene glycol) diacrylate (PEG-DA) has also been employed to immobilize or encapsulate various types of living cells in order to study multistep cellular behaviors [8-10].

Compared with conventional crosslinking methods, photo-crosslinking can enable simple, rapid, and effective production of biocompatible polymers. Photo-crosslinking has the following advantages [5, 11, 12]: first, polymerization of free radical chains is easily initiated by low intensity light irradiation; second, the process of gelation can be temporally and spatially controlled by altering the exposure conditions to light irradiation; third, rapid production of

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biomaterial can be achieved by in situ gelation for only a short photo-polymerization time; and fourth, photo-initiated polymerization occurs at physiologically sustainable temperatures and pH ranges, allowing the polymerization to incorporate living cells and biological molecules without damaging them.

Photo-polymerization can be easily driven by a variety of photo-initiators, which release free radicals upon exposure to specific light irradiations [12-14]. However, inactivated residues or byproducts of initiators after photo-polymerization may have cytotoxic effects on cells embedded in hydrogels, which would be a critical issue [1, 5, 12]. Therefore, it is very important to study whether the concentrationof a given photo-initiatoris correlated with cellular cytotoxicity.

To address this issue, the viability of cells embedded in gelatin methacrylate (GelMa) microgels (a common photo-crosslinkable hydrogel) was investigated as a function of photo-initiator concentration. Cell-encapsulated GelMa microdroplets were generated using a flow-focusing microfluidic device. Then, these droplets were UV-crosslinked using 2-hydroxy-1-[4-(hydroxyethoxy)phenyl]-2-methyl-1-propanone, a water-soluble photo-initiator widely used in a variety of cell types.

2. Experimental

2.1 Synthesis of gelatin methacrylate

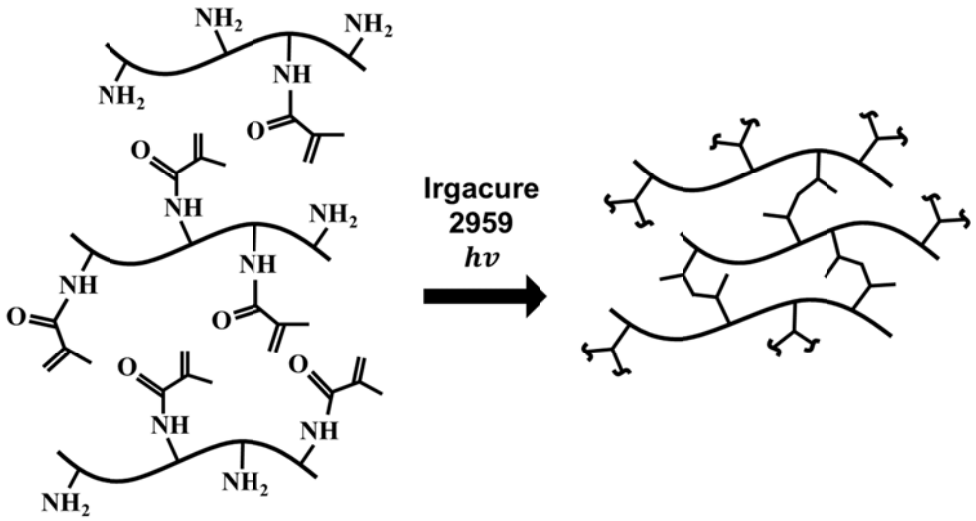
Photo-polymerizable GelMa was synthesized by reacting gelatin with methacrylamides, thus replacing the amine groups of gelatin with the methacrylic functional groups. In this study, gelatin (type A, bloom strength of 300; Sigma Aldrich, St. Louis, MO, USA) was isolated from porcine skin. While stirring at 50^{o}C, gelatin (5 g) and 4-(dimethlyamino)-pyridine (0.5 g; Sigma Aldrich) were solubilized in dimethyl sulfoxide (Sigma Aldrich). Then, glycidyl methacrylate (2 mL; Sigma Aldrich) was added to the mixture at a constant rate of 0.5 mL/min. After allowing the reaction to proceed for two days in a dry N_2 gas atmosphere, the reaction products were purified using dialysis membranes (molecular weight cut off 12,000–14,000 Da; Sigma Aldrich) with deionized water for one week, at a constant temperature of 40 ^{o}C. Finally, after freeze-drying the purified solution, a white solid consisting of GelMa was obtained.

2.2 Flow-focusing microfluidic device

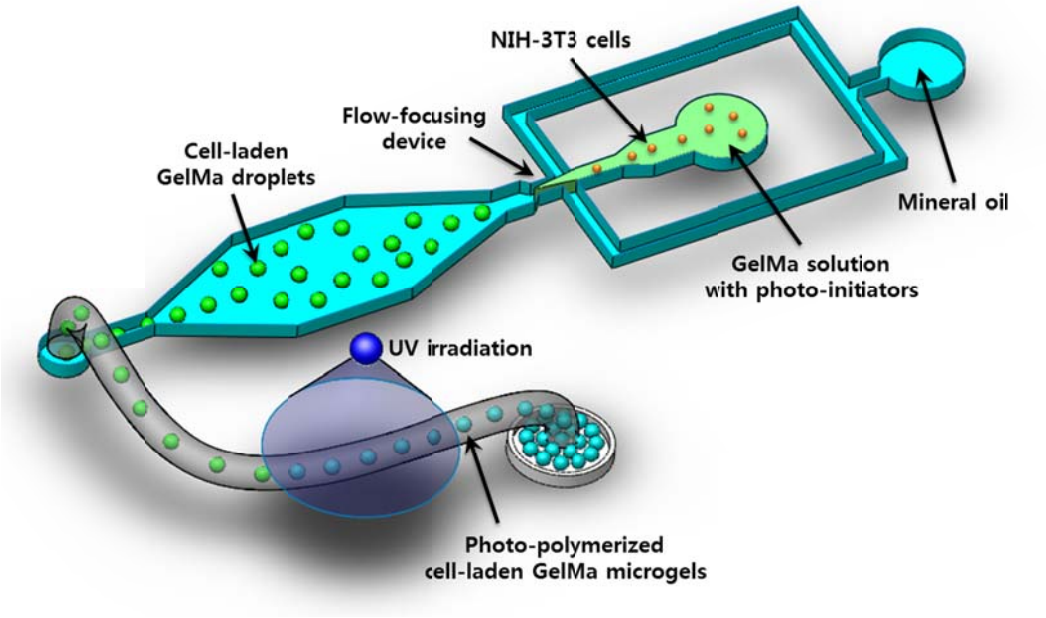
Using standard photolithography, an SU-8 master mold was prepared by etching a preset design of microchannels onto a silicon wafer. The silicone elastomer base and the curing agent (Sylgard184 silicone elastomer kit; Dow Corning, Midland, MI, USA) were mixed at a ratio of 10 to 1. The mixture was poured onto the SU-8 master mold, and then cured at 100°C for one hour. After separating the polydimethylsiloxane (PDMS) part from the SU-8 master mold, holes were perforated in the mold to create inlets and outlets. The flow-focusing microfluidic device was then prepared by permanently bonding the PDMS component with a glass slide under plasma treatment (Harrick Plasma, Ithaca, NY, USA).

2.3 Generation of GelMa microdroplets

Fig. 1a shows a schematic feature of the microfluidic device that was used to generate microdroplets. The freeze-dried GelMa macromer was dissolved in phosphate-buffered saline (pH 7.4) at 60°Cto prepare a 5 wt% GelMa prepolymer solution. Mineral oil was then prepared by adding a 20 wt% emulsifier (Span 80, Sigma Aldrich). To characterize the effects of photo-initiator concentration on cell viability during the photo-polymerization process upon UV exposure, different concentrations (0.2, 0.5, 0.7, and 1.0 wt%) of the photo-initiator (2-hydroxy-1-[4-(hydroxyethoxy)phenyl]-2-methyl-1-propanone; Irgacure 2959; Ciba Specialty Chemicals, Basel, Switzerland) were added into the GelMa prepolymer solution.



(a)



(b)

Fig. 1. (a) Photo-polymerization reaction of GelMa driven by a photo-initiator; (b) schematic description of generation of photo-polymerized GelMa microgels in a flow-focusing microfluidic device. Flow-focusing channel width and height = 100 μm.

Prepared GelMa prepolymer solution, including the appropriate concentration of photo-initiator, was perfused into the microfluidic channels at a flow rate ranging from 100 to 300 μL/h. Mineral oil was then introduced into the device at a flow rate of 1000 μL/h. Syringe pumps (KD Scientific Inc., Holliston, MA, USA) were used to precisely control the infusing flow rates. In the flow-focusing device, the GelMa prepolymer solution was used as the dispersed phase, while mineral oil was used as the continuous phase. When the two immiscible fluids (GelMa prepolymer solution and mineral oil) came into contact in the flow-focusing channels, GelMa microdroplets were generated by the shearing effect resulting from contact with the continuous phase. The generation of GelMa microdroplets was monitored and recorded using an inverted optical microscope (Olympus, Tokyo, Japan).

2.4 Crosslinking of GelMa microdroplets

The photo-polymerization of GelMa microdroplets was conducted using UV light treatment. The schematic polymerization process is outlined in Fig. 1b. Aqueous GelMa microdroplets, generated with Tygon tubing (inner diameter = 0.3 mm; thickness = 0.25 mm) were exposed to UV irradiation for 5 min, and cured at an intensity of 0.85 W/cm^2, using a UV lamp (OmniCure S2000; Lumen Dynamics, ON, Canada). The resultant crosslinked GelMa microgels were collected, harvested by centrifugation, and rinsed with phosphate-buffered saline (PBS).

2.5 Cell encapsulation and viability test

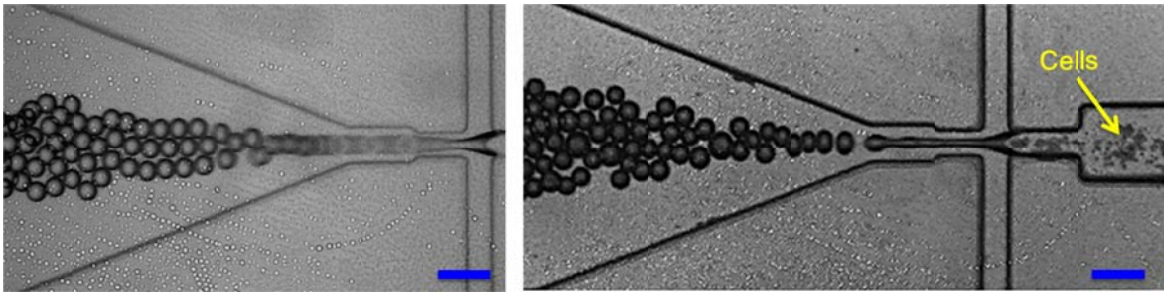
NIH-3T3 cells (mouse embryonic fibroblasts) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 1 % penicillin-streptomycin (Invitrogen) and 10 % fetal bovine serum (Invitrogen). Cell incubation was performed in a humidified 5 % CO_2 environment at 37 °C. Cells were passaged every three days, and medium was changed every 24 h. Prepared NIH-3T3 cells (10^7 cells per mL) were then premixed with 5 % GelMa prepolymer solutions.

Fabricated cell-laden microdroplets were photo-crosslinked, and the viability of NIH-3T3 cells was investigated one day later using a Live⁄Dead® Viability Cytotoxicity Kit (Invitrogen). This kit employs calcein-AM (0.5 μL/mL; green stain for live cells) and the ethidium homodimer-1 (2 μL/mL; red stain for dead cells) to allow visualization of live and dead cells. Cells were stained in the GelMa microgels for 15 min at 37 °C. After rinsing with PBS, cell viability was observed using a fluorescence microscope. The numbers of live and dead cells were counted for ten different microgels at each concentration of photo-initiator. Measured results are expressed as means and standard deviations. Statistical analysis was performed using the ANOVA test, with photo-initiator concentration set as a main effect and cell viability set as the dependent variable.

3. Results and discussion

Upon introduction of the two immiscible fluids into the microfluidic device, GelMa microdropletswere rapidly generated in the flow-focusing channel. In a flow-focusing microfluidic system, shear stress between the two phases could play an important role in the physical deformation of the dispersed phase into microdroplets. Different outcomes were observed in the flow-focusing microfluidic system, depending on whether cells were present in the dispersed phase. At GelMa prepolymer solution flow rates less than 200 μL/h, microdroplets were uniformly generated, regardless of the presence of cells. However, increasing the flow rate of GelMa prepolymer solution greater than 200 μL/h caused cell-laden microdroplets to be generated in an unstable manner, as shown in Fig. 2. This instability was exacerbated by the increased viscosity of the dispersed phase upon the addition of cells, which resulted in an unstable jetting length of GelMa prepolymer solution and non-uniform microdroplets.

UV irradiation initiated the process of radical chain polymerization by photo-initiators in the microgels. The total amount of energy to which the microgels were exposed during the experimentwas25.5 J/cm^2, which was the minimum amount able to maintain the spherical shape of 5 wt% GelMa microdroplets. A 0.2 wt% concentration of photo-initiator, incorporated with the applied UV irradiation energy, was chosen as the minimum threshold in this study. When the concentration of photo-initiator was less than 0.2 wt%, only weak crosslinking networks were formed; consequently, microgels ruptured during swelling.



(a) (b)

Fig. 2. Microdroplet generation in flow-focusing microfluidic channels using GelMa prepolymer solutions (a) without cells and (b) with cells; scale bar = 200 μm.

Cell-laden GelMa microdroplets, containing different concentrations of photo-initiator (0.2, 0.5, 0.7, and 1.0 wt%) were photo-polymerized under a UV light source. The influence of photo-initiator concentration on cell viability was assessed using a fluorescent labeling strategy. Fig. 3 shows fluorescence microscope images of live (green) and dead (red) cells encapsulated in the GelMa microgels. Cells were cultured for one day to determine the effects of photo-initiator concentration on their viability. At 0.2 wt%, the photo-initiator did not exert any cytotoxic effects after photo-crosslinking. However, cell viability was somewhat impaired when the photo-initiator concentration was increased to 0.5 wt%. Photo-initiator concentrations above this amount led to a continuous increase in cytotoxicity. At 1.0 wt%, almost no live cells were observed under the fluorescence microscope.

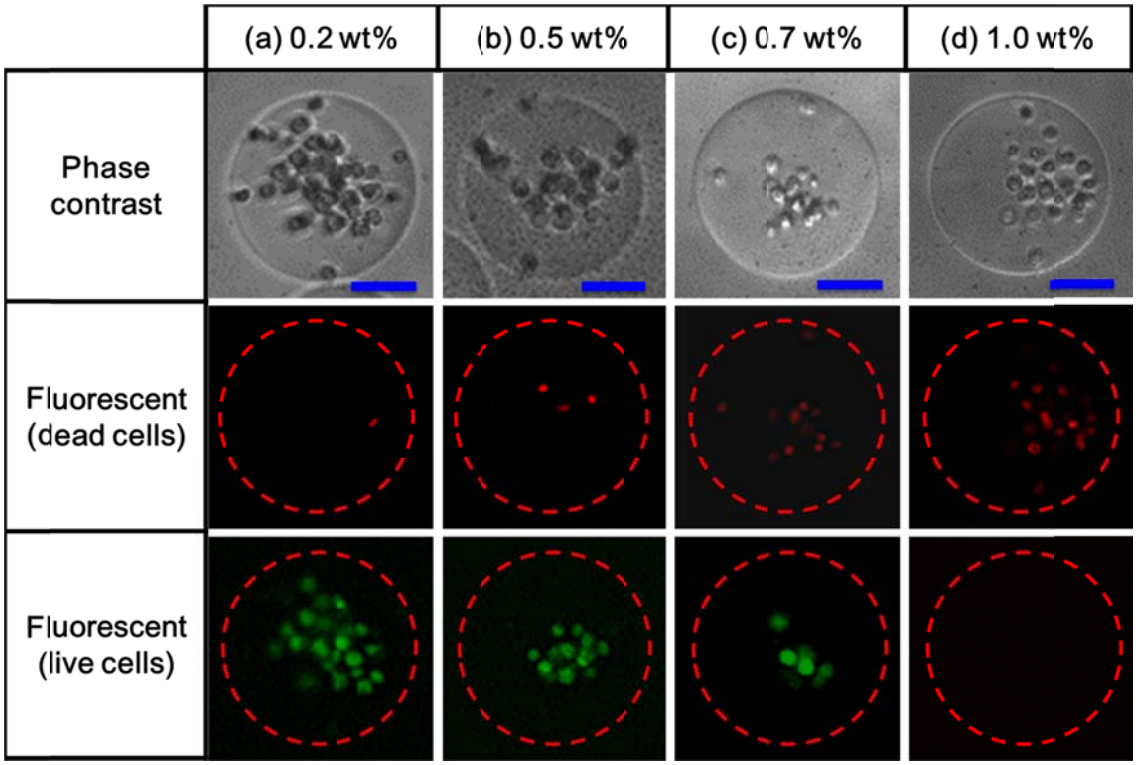


Fig. 3. Phase contrast and fluorescence images of live (green) and dead (red) NIH-3T3 cells embedded in GelMa microgels one day after photo-crosslinking; (a) 0.2 wt%, (b) 0.5 wt%, (c) 0.7 wt%, and (d) 1.0 wt% photo-initiator, respectively. Cell viability was evaluated by fluorescence labeling with calcein-AM (green, live cells) and ethidium homodimer-1 (red, dead cells). Scale bar = 30 μm.

Fig. 4 shows a quantitative assessment of the number of viable cells encapsulated per microgel. Based on the results shown in Fig. 3, a negative correlation was observed between photo-initiator concentration and cell viability. At 0.2 wt%, the photo-initiator was associated with a high survival rate, 87.0 ± 5.2 %. However, increasing the concentration of the photo-initiator in the GelMa prepolymer solution decreased cell viability. At a photo-initiator concentration of 1.0 wt%, cell viability drastically decreased to 3.5 ± 3.0 %. This phenomenon appears to result from a highly cytotoxic effect, exerted by high concentrations of the photo-initiator, on the encapsulated cells.

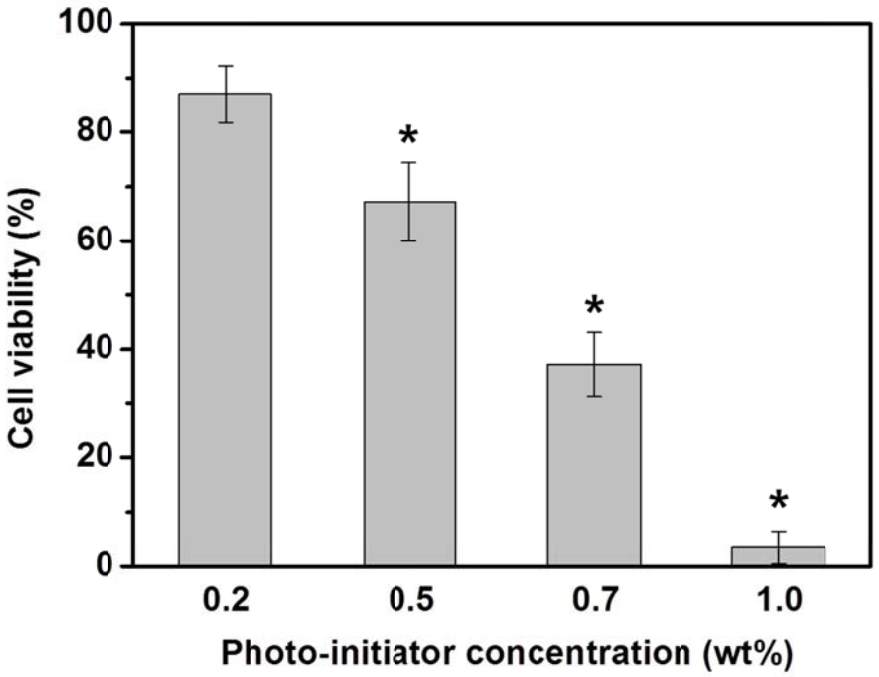


*Fig. 4. Viability of cells embedded in GelMa microgels, one day after photo-crosslinking, as a function of photo-initiator concentration (*p<0.001).*

The observed cytotoxicity of the photo-initiator might result from inactivated residues in the photo-initiator, which presumably remains in the cell-laden GelMa microgel after photo-polymerization. Even though the photo-crosslinked microgels were rinsed three times, remaining photo-initiator apparently still existed in the microgels. This conclusion is based on the correlation results, and suggests that the cytotoxic effects on cells of the photo-initiator are due to its hydrophobic structure. In addition, byproducts formed as a result of photo-initiator chemical dissociation might also contribute to the decreased cell viability.

4. Conclusion

Photo-polymerized GelMa microgels were produced using a flow-focusing microfluidic device. Cells were encapsulated in microgels containing different concentrations of photo-initiator, and the resultant cell viability was determined at each concentration. At a concentration of 0.2 wt%, the photo-initiator enabled stable gelation without affecting cell viability. However, at concentrations greater than 0.5 wt%, the photo-initiator was obviously cytotoxic, as evidenced by the significant decreases in cell viability. These decreases in viability are probably due to residues and byproducts of photo-initiators.

We anticipate that the results presented here will be valuable for achieving successful biomedical applications using microgels and photo-initiators.

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