Optimizing Biomaterials for iPSC-Derived Engineered Heart Tissue Models

Huanzhu Jiang

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Optimizing Biomaterials for iPSC-Derived Engineered Heart Tissue Models

By

Huanzhu Jiang

A thesis presented to the McKelvey School of Engineering of Washington University in St. Louis in partial fulfillment of the requirements for the degree of Master of Science

May 2021

St. Louis, Missouri
Dedication

I want to dedicate this thesis to my friends and family, especially to my parents for their unconditional support, patience and love.
Acknowledgements

I would like to express my deepest appreciation to my advisor, Dr. Nathaniel Huebsch, who has provided endless patience and tireless guidance to me during my every step in the master research.

I would also like to thank my all my lab mates, especially Jingxuan Guo, Xiaohong Tan, Soore Oguntuyo, Ghiska Ramahdita, Sydney Neal and David Schuftan. Thank you for your support and companion.

Finally, I would like to acknowledge my committee members, Dr. Rutz and Dr. Guan. Thank you for taking time to read this thesis and attend my defense.

Huanzhu (Harper) Jiang
Washington University in St. Louis
May 2021
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Abstract

Optimizing Biomaterials for iPSC-Derived Micro Heart Tissue Models

By

Huanzhu Jiang

Master of Science in Biomedical Engineering

Washington University in St. Louis, 2021

Research Advisor: Professor Nathaniel Huebsch

Hypertrophic Cardiomyopathy (HCM) is the most common inherited cardiovascular disease, where environmental forces and genetic mutations have been heavily linked to heart failure or sudden cardiac death in young people. However, how genetic mutations and environmental forces contribute to HCM phenotype are not well defined. To study the mechanisms and investigate the potential treatments of this disease, it is important to build a model to simulate mechanical loading and recreate micro-environment of cardiomyocytes. In vivo, surgical maneuvers like transaortic constriction allow the study of overload, but these approaches are low throughput and it is difficult to precisely control overload. In vitro models for mechanical overload have shown differences in results. In this study, I contributed to the development of an “overload enhanced iPSC-micro heart muscle (iPSC-μHM)” model, and optimized substrate preparation to improve quantitative analysis of tissue contractility and calcium handling. I also explored the possibility of using synthetic extracellular matrix on optimization of μHM model. Gelatin methacryloyl (GelMA), one of the most common photo-crosslinkable hydrogels, is applied to encapsulate cells. Results show successful reduction of autofluorescence during process of surface modification on substrates. Moreover, I showed the feasibility of cell encapsulated in GelMA, which can potentially reduce
the number of cells required to form iPSC-μHM, and to simulate different stiffnesses of micro

tissue by varying concentrations of GelMA and photo-initiators.
Chapter 1: Introduction

Hypertrophic Cardiomyopathy (HCM) is the most common inherited cardiovascular disease happening to about 1 out of every 500 people, leading to cardiac arrhythmia, heart failure, and sudden cardiac death (Maron & Maron MS., 2013; Maron., 2018). Although at a lower incidence, HCM is the most important cause of sudden cardiac death in young athletes (Makarov., 2020). Genetic studies have shown that mutations in the contractile sarcomere of cardiomyocytes are the most common cause to HCM by far, and the most frequent mutation that account for almost 50% HCM patients is found in Myosin Binding Protein C (MYBPC3) (Carrier et al., 2015; Marian & Braunwald, 2017). While genetic defects of cardiac myocytes play the most critical role in leading to HCM, the penetrance of HCM phenotypes varies highly in different individuals with genetic mutations, and HCM cases without any sarcomere mutations were reported (Ingles et al., 2017; Lorenzini et al., 2020). This indicates that environmental factors, such as mechanical overload caused by change of blood pressure and change of myocardial stiffness may also trigger changed in HCM phenotypes.

Different in vivo and in vitro models have been developed to study HCM mechanisms, including potential synergy between mechanical loading and HCM mutations in order to provide future perspectives for HCM treatment (Guo & Huebsch, 2020; Mosqueira et al., 2019). In vivo models have showed their crucial role in studying mechanisms with their advantages, such as tractable genetic manipulation, but the difficulty in controlling mechanical overload, physiological differences between humans and animals, and time-consuming problems (Barefield et al., 2015; Milani-Nejad & Janssen, 2014) cannot be disregarded. Thus, in vitro systems for studying synergy between mechanical overload and HCM genetic mutations are needed.
Previous *in vitro* HCM models using single cell, 2D and 3D format showed conflicting results which urge the need for more accurate and standard model. Few of these studies investigated potential trigger – synergy between mechanical overload and genetic mutations to HCM phenotypes. (Birket et al., 2015; Cohn et al., 2019; Davis et al., 2016; Lan et al., 2013; Ma et al., 2018; Ribeiro et al., 2017).

A medium-throughput iPSC-micro heart muscle (iPSC-μHM) model was developed recently (Guo et al., 2020), which shows high potential to study synergetic effects of mechanical overload and genetic mutations on HCM phenotype by applying heterozygous MYBPC3 iPSC cell line. However, some limitations of this iPSC-μHM model were shown, such as increase in autofluorescence (Lee et al., 2013) during substrates preparation process, which affects the quantification of calcium transient (*Figure 1*).

Glutaraldehyde (GA) can cause high autofluorescence defined as unwanted fluorescence sources. The unwanted visible emitters are generated during the process of reaction between GA and proteins or peptides. A study indicated that the existence of secondary amine in the molecule plays a crucial role in the formation of emissive species, and pH can also influence the generation of different emitters with different colors, for example, a yellow emitter can be formed at higher pH (Lee et al., 2013). Based on the possible mechanism, adjusting pH during reaction process or introducing a new chemical at the end of GA reaction by reacting with the fluorophores are potential ways to reduce or eliminate autofluorescence. However, pH change and a new added chemical may be toxic to cells, which are not practical in the application into optimization of the iPSC-μHM model. A simpler and effective way was proposed in this study.
Furthermore, engineered hydrogel is widely used in tissue engineering for tissue production and regeneration, which is the optimal material for 3D \textit{in vitro} models due to its biocompatibility, biodegradability, and hydrophilicity (Graham, 1998). Gelatin methacryloyl (GelMA) is one of the most common photo-crosslinkable hydrogels used for tissue regeneration due to its optimal biological environment and fast photo-crosslinked process, and the visible light photo-crosslinking process makes GelMA possible for cell encapsulation with fewer biosafety problems, such as DNA damage and cancer (Noshadi et al., 2017). The mechanical properties and degradation rate of GelMA hydrogel can be modified by varying the methacrylation degree, photo-initiator concentration, temperature, and photo-polymerization time (Noshadi et al., 2017; Van et al., 2000). Here, GelMA was tested using C2C12s by varying photo-initiator concentration, GelMA concentration, and photo-polymerization time to investigate a better polymerization condition which can be potentially applied into the iPSC-μHM model.
The goal of this study is divided into two parts based on the iPSC-μHM model – investigate an effective way to reduce autofluorescence during reaction between glutaraldehyde (GA) and APTES, and introduce GelMA to encapsulate cells to investigate the potential to lower cell densities and simulate varies tissue stiffnesses. This study shows a simple way to reduce autofluorescence, and possibly better condition in crosslinking GelMA for cells encapsulation that can potentially be uses in the iPSC-μHM model in the future.

![Image of autofluorescence caused by GA on the substrates surface.](image)

**Figure 1.** Autofluorescence caused by GA on the substrates surface.
Polydimethylsiloxane (PDMS) Substrate Preparation

To simulate different mechanical overloads, Sylgard 184 and Sylgard 527 were mixed at different ratios to manipulate and control substrate stiffness (Palchesko et al., 2012). Sylgard 184 (Dow Corning; Midland, MI) elastomer was mixed in a ratio of 1:10 (base to curing agent), and Sylgard 527 was mixed in a ratio of 1:1 (base to curing agent). The whole fabrication process followed a previous method (Guo et al., 2020). To study the surface modification situation, Sylgard 184 was only applied here in 24 well plates.

“Dog-Bone-Shaped” Stencil Molds Fabrication

The “dog-bone-shaped” stencil molds (Figure 2A) were fabricated and assembled following a previous protocol (Guo et al., 2020). PDMS (Sylgard 184, 2 MPa) was poured over predefined 3D-printed molds (MicroFine Green; Protolabs, Inc., Maple Plain, MN) designed by SolidWorks (Dassault Systèmes SE, Vélizy-Villacoublay, France). The thickness of each knob of each “dog-bone-shaped” stencil mold is 1 mm, and each mold has two 1 mm × 1 mm knobs, which are connected by a 1 mm × 200 μm shaft. The “dog-bone-shaped” stencil molds were cured at 60°C overnight. To avoid tissue attachment to stencils surface, stencils were soaked in 10% F68 Pluronic for at least 45 min at room temperature followed by 3 washes using dPBS. Then stencils were soaked into methanol and attached onto substrates. The attachment between stencils and substrates was achieved through incubation for 2 hours at 60°C to evaporate methanol. The assemble devices were sterilized with 70% sterile ethanol for at least 3 hours. Upon sterilization, all the ethanol was
aspirated, and 5 μg/mL fibronectin was seeded inside stencils (3 μL/“dog-bone-shaped” stencil) for 3 hours followed by 3 washes using dPBS before seeding cells.

**Substrates Surface Modification**

To achieve strong mechanical bonding between tissue and substrates, surface modification was applied to get covalently ECM protein (bovine plasma fibronectin, FN; Sigma; Millipore Sigma; St. Louis, MO)–grafted substrates following the previous protocol (Figure 2B) (Guo et al., 2020). Cured substrates were firstly treated with plasma oxidization (Harrick plasma, Ithaca, NY; pressure/flow rate: 580–680 mTorr) for 90 s at high power (30 W). Upon plasma treatment, amino silane chemistry was conducted by adding (3-aminopropyl) triethoxysilane silane (APTES; 5% v/v in methanol, Sigma) onto the surfaces of each substrates immediately. The reaction was conducted at 60°C for 1 hour to graft amine groups to substrate surfaces. After 1 hour of APTES reaction, substrate surfaces were washed with methanol for three times and 5 min for each time to remove unreacted APTES. To investigate the way to reduce strong autofluorescence caused by Glutaraldehyde (GA), substrates were incubated in Dulbecco’s phosphate buffered saline (dPBS) for 24 hours at room temperature after methanol washes. GA (Thermo Fisher Scientific; Waltham, MA, 2.5% v/v in dPBS) reaction was performed on the shaker for 2 hours at room temperature to act as an amine-reactive crosslinker, followed by 3 washes using dPBS (with or without 24 hours of dPBS incubation) before FN reaction. FN (15 μg/mL in dPBS) reaction was applied on the shaker for at least 30 min at room temperature. After FN grafting, substrate surfaces were washed for 3 times with dPBS (5 min each time) and then were washed with deionized water for another 3 times (5 min each time). Then ethanolamine hydrochloride (EtOH-Amine; Thermo Fisher Scientific, 2.5% w/v in dPBS) was applied to the substrate surfaces to quench unreacted GA
followed by 3 washes using dPBS. Fluorescent beads (1.06 μm diameter, Polysciences, Inc., Warrington, PA) were applied at the top of PDMS substrates through spinning coating to estimate the contraction of iPSC-μHM by tracking their displacements.

Figure 2. iPSC-μHM device fabrication and surface characterization (Guo et al., 2020). (A) Schematic of “dog-bone-shaped” stencil assembly to substrate and tissue formation. Cells are seeded inside “dog-bone-shaped” stencils, and compact to form tissues; Formed tissues collapse and are detached from knob regions over time. (B) Schematic of substrate surface modification. APTES is applied and leaves amines on the surface after plasma oxidation. Reactive aldehydes are grafted onto amines after GA reaction.

Fibronectin Bioactivity Characterization

To make sure the bioactivity of FN did not get changed with the 24 hours of dPBS incubation, a previous antibody-based assay (García et al., 1999) was performed. Fibronectin antibody clone hFN7.1 (Developmental Studies Hybridoma Bank, Iowa City, IA) was applied to determine and quantify the bioavailability of integrin-binding epitopes in the substrate-adsorbed FN. Pure Sylgard 184 were poured into 24 well plates and got fully crosslinked, followed by plasma oxidation and APTES reaction. After either GA added immediately or 24 hours later after APTES reaction, different concentrations of human FN (Sigma, 0, 1.25, 5 and 20 µg/mL in dPBS) were grafted to the substrate surfaces followed by 3 washes using dPBS and 3 washes using deionized water. Goat serum (Invitrogen, Carlsbad, CA) and bovine serum albumin (BSA, Sigma) were
reconstituted into 3% respectively with 0.1% Triton-X-100 (Thermo Fisher Scientific, diluted in dPBS) to be used as the blocking buffer. The blocking buffer was added to the substrate surfaces and the reaction was performed at room temperature for 45 min. After blocking reaction, hFN7.1 (2 μg/mL in blocking buffer) binding reaction was conducted for 1 hour at room temperature following the primary antibody incubation assay. 3 thorough washes using 0.1% Triton-X-100 were followed. Li-COR IRDye 800cw secondary antibody (LI-COR Biosciences, Lincoln, NE, 100 ng/mL in Milli-Q water) was applied to react for 1 hour at room temperature, followed by 0.1% Triton-X-100 wash buffer washes for 3 times. Then the substrate surfaces were scanned with a Li-COR Odyssey CLx scanner (LI-COR Biosciences), and hFN7.1 fluorescence intensity was measured with Image Studio Lite Version 5.2.5. (Guo et al., 2020).

**iPSC-μHM Calcium Transient**

iPSC cell culture, cardiomyocyte differentiation, iPSC-μHM formation on substrates, and the analysis of iPSC-μHM contractility and calcium transient were conducted to study cardiac physiology following the protocol (Guo et al., 2020). The genetically encoded calcium indicator, GCaMP6f were applied to visualize calcium dynamics on substrates with different stiffnesses (afterload) and study stiffness effects on calcium handling (Figure 3) (Guo et al., 2020; Huebsch et al., 2015). At least 3 regions without fluorescent beads were selected for each video imaged at 100 frames per second to analyze calcium handling to prevent the effects of fluorescent beads due to the condition that fluorescent beads and the calcium signal are both green fluorescence. A custom MATLAB code (Ma et al., 2018) was used to separate and analyze individual waveforms to obtain calcium transient values. Fridericia’s formula (Fridericia, 2003) was used to reduce the
effects of different spontaneous beat rate on calcium transient. GCaMP intensity (amplitude) over time can be integrated to get calcium flux.

Figure 3. GCaMP6 signal traces (Guo et al., 2020). (A) Representative traces of iPSC-μHM on the 5 kPa substrate on Day 16. (B) Representative calcium transient with upstroke and decay times noted.

**Synthesis and Preparation of GelMA**

GelMA was prepared and synthesized on the basis of previous protocols (Figure 4A) (Kerscher et al., 2017; Nichol et al., 2010; Van et al., 2000; Zhu et al., 2019). Gelatin (Type B, bovine skin, Sigma) was mixed into dPBS in a ratio of 5% (w/v) on the stir play at 60°C to ensure gelatin powder got fully dissolved. Methacrylic anhydride (MA, Sigma, 15% w/v in gelatin solution) was added into fully dissolved gelatin solution constantly at the rate of 0.5 mL/min. The MA reaction was performed at 60°C for 2 hours on the stir play. Then the reaction was stopped with 60°C dPBS. The volume of dPBS for stopping the reaction is 5 times of original fully dissolved gelatin solution. The mixture was put into 10k MWCO dialysis tubes (Thermo Fisher Scientific) and dialyzed at
60°C for 7 days using deionized water. The GelMA solution was lyophilized for 5-7 days and stored in the -80°C freezer for use.

**Preparation and crosslinking of GelMA Hydrogel**

GelMA hydrogel is crosslinked through photoinitiated method by combining Eosin Y photoinitiator with triethanolamine (TEA) and N-vinypyyrolidone (NVP). Free radicals are generated from the photoinitiator which initiates the chain polymerization of the methacryloyl groups (Figure 4B) (Noshadi et al., 2017). Visible light UV was used here, which is a more suitable light source to crosslink GelMA, because studies have shown that UV light might potentially cause DNA damages in the cells (Kerscher et al., 2017).

Lyophilized GelMA was mixed and dissolved into dPBS at a concentration of 150 mg/mL (15%). To investigate a proper concentration of different components in the crosslinker for GelMA crosslinking, different 10X crosslinkers were prepared containing different concentrations of triethanolamine (TEA, Sigma, 1-15% v/v), Eosin Y (Sigma, 0.1-2mM), 369 mM 1-vinyl-2-pyrrolidinone (NVP, Sigma). Eosin Y was first dissolved in Dimethyl sulfoxide (DMSO, Sigma) to reach 100mM. The precursor solution was prepared by combining 15% lyophilized GelMA solution, 10X crosslinker and then dissolving them in dPBS to reach 7.5% or 10% GelMA precursor solution. Then the precursor solution was crosslinked by being exposed to LED light (Otdair RGB LED Floodlight) for 2.5-20 min to get GelMA hydrogel.
Figure 4. Synthesis and crosslinking of GelMA hydrogel (Zhu et al., 2019; Noshadi et al., 2017). (A) Methacryloyl groups are grafted onto the gelatin backbone after the reaction of gelatin and methacrylic anhydride. (B) Schematic of photo-crosslinking reaction of GelMA to form hydrogel network. Photoinitiator (Eosin Y) and Organic initiators (NVP, triethanolamine) produce free radicals to initiate chain polymerization to crosslink methacryloyl groups from one polymer chain to another polymer chain.

**Mechanical Compression Testing of GelMA**

7.5% or 10% GelMA precursor solutions combined with different concentrations of Eosin Y (10, 20 and 50 μM) were prepared following the protocol as described before. 90 μL GelMA precursor solution was put onto an acrylic plate, then covered with another acrylic plate, and two plates were separated using 1mm thick glass coverslips to make sure the surface of GelMA hydrogel is flat and the thickness of each hydrogel is 1mm. GelMA precursor solution was exposed to LED light for 10 or 20 min. Stiffness of hydrogel was measured using ElectroForce 3200 (TA Instruments) with 225 newton load cell.
Compaction study of C2C12s Encapsulated in GelMA

7.5% or 10% GelMA precursor solutions combined with different concentrations of Eosin Y (10, 20 and 50 μM) were prepared following the protocol as described before. Cells were first mixed with GelMA precursor solution, then the mixture was added in 96 well plates (2 μL/droplet) or seeded the mixture in “dog-bone-shaped” stencil (3 μL/ “dog-bone-shaped” stencil) separately, and then exposed them to LED light for 2.5 or 5 min to crosslink. The areas of GelMA were measured on Day 0, Day 6, Day 10, and Day 15.

Micro Tissue Formation

C2C12 fibroblasts were singularized using 0.05% Trypin and resuspended into Dulbecco’s Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) or GelMA precursor solutions before seeded to “dog-bone-shaped” stencils. Upon pure cells were seeded into stencils, incubation at 37°C for 2 hours should be performed before adding DMEM with 10% FBS into each well. DMEM with 10% FBS was added immediately when GelMA was crosslinked. Cells will self-assemble with GelMA to form 3D tissue. The cells densities of $2.5 \times 10^7$, $5 \times 10^7$, $8 \times 10^7$ cells/mL were applied.
Chapter 3: Results of Optimization of Surface modification and GelMA Crosslinking

**Reduction of Substrates Autofluorescence**

Since pH adjustment and addition of new chemicals are not suitable for the optimization, we proposed a way where we added a period of incubation in dPBS after APTES grafting before GA reaction, which was proven an effective way to reduce autofluorescence. GA reacting 24 hours after APTES grafting resulted in significant lower autofluorescence (Guo et al., under review). The prolonged incubation in dPBS reduced the activity of APTES grafted on the substrates surface and washed out unreacted APTES, thus reduced autofluorescence caused from reaction of APTES and GA, which can also reduce toxicity from the unreacted silane.

**Changes of Fibronectin Bioactivity**

The characterization of FN binding affinity between different surfaces using FN antibody binding assays indicated that the dPBS incubation did not alter FN bioactivity, when lowering the substrates autofluorescence (Guo et al., under review). When hFN concentration increased, the hFN 7.1 fluorescent intensity increased, no significant difference was found between two surfaces.

**iPSC-μHM Calcium handling**

The effects of afterload on iPSC-μHM Calcium handling were investigated and data were shown (Figure 5) (Guo et al., 2020). Higher afterload causing higher calcium and net flux was observed (Figure 5A, B). However, lowest upstroke duration (Figure 5C) and calcium decay value (Figure 5D) were captured on iPSC-μHM on 15 kPa substrates. Calcium decay times on Day 16 were significantly larger than times on Day 6. No significant differences were indicated on calcium amplitude on substrates with different stiffnesses on Day 9. But stiffer substrates tended to lead to
increase of calcium amplitude. On Day 9, iPSC-μHM on 30 kPa substrates has significantly higher integrated calcium flux than iPSC-μHM on 5 and 15 kPa (Figure 5B). A significant longer upstroke time ($p=0.008$, Figure 5C) calcium decay times ($p=0.0005$, Figure 5D) of iPSC-μHM on 30 kPa substrates than other two groups. On Day 16, stiffer substrates led to significantly higher calcium amplitude and calcium flux (Figure 5A, B).

Figure 5. iPSC-μHM calcium transient on substrates with different stiffnesses on Day 9 and 16 (Guo et al., 2020). (A) Stiffer substrates led to higher calcium amplitude ($p=0.035$) on Day 9. On Day 16, no significant differences were observed in calcium amplitude between iPSC-μHM on 5 and 15 kPa substrates ($p=0.560$), while 30 kPa substrates led to significantly higher value compared to other two groups respectively ($p=0.0002$, $p=0.0012$). (B) On Day 9, no significant differences of integrated calcium flux between 5 and 15 kPa conditions were indicated ($P=0.316$). The 30 kPa condition had a significant higher flux than 5 kPa ($p=0.003$) and 15 kPa ($p=0.043$) conditions. On Day 16, 5 kPa and 15 kPa conditions showed no significant differences between them ($p=0.615$), but they both showed significantly lower flux than the 30 kPa condition ($p<0.0001$). (C) Upstroke duration of the 15 kPa condition showing the lowest value was significantly lower than the 30 kPa condition on both Day 9 ($p=0.008$) and 16 ($p=0.002$). (D) The decay time indicated by calcium $\tau_{75}$ showed that the 30 kPa condition was significantly higher than the 15 kPa condition ($p=0.0005$) on Day 9. On Day 16, decay time of both 5 and 15 kPa conditions was significantly lower than the 30 kPa condition ($p<0.0001$). The 5 kPa condition had significantly longer decay time than the 15 kPa condition on Day 16 ($p=0.009$). ****, ***, **, and * indicate significant difference $p < 0.0001$, 0.001, 0.01, and 0.05, respectively.
**Photo-initiator Concentrations for GelMA Crosslinking**

Eosin Y at a concentration higher than 100 μM and TEA at a concentration higher than 1.5% v/v are toxic to human mesenchymal stem cells (hMSCs) (Bahney et al., 2011). Based on this, I tested 5-100 μM Eosin Y and 0.05-1.5% TEA, different concentrations of GelMA (1.5%, 5%, 7.5% and 10%) and different photoinitiation times using total volume of 3 μL precursor solution for each sample (Table 1). We used pipette tips to gently poke the surface of each sample after exposed to LED light for 2.5 min to observe if GelMA was crosslinked. By trying different concentrations of Eosin Y, TEA and GelMA, 10, 20 and 50 μM Eosin Y, 1.5% v/v TEA, and 7.5% and 10% GelMA in the final precursor solution were applied in the further study.

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Table 1. Design and results of different input parameters for GelMA crosslinking testing.

However, GelMA with higher volume could not crosslink with 2 min of photo-polymerization time. The time for GelMA to crosslink completely is related to thickness of GelMA, but the this relationship remains to be investigated.
Mechanical Properties of GelMA

Based on previous study, higher volume of GelMA needs longer time to crosslink. To make sure GelMA hydrogels used for mechanical testing can crosslink, we used the same observation method here – tips were applied to gently poke the surface of each sample after the certain time of exposure to light. At least 8.5 min light exposure was needed for 90 µL 7.5% GelMA to crosslink.

Higher concentration of Eosin Y and higher concentration of GelMA led to significantly higher stiffness (Figure 6A). However, 10% GelMA did not generally cause a significant increase of stiffness compared to 7.5% GelMA with the same crosslinking conditions. There are no significant differences among 5 conditions, which were: 10% GelMA_10 µM Eosin Y, 10% GelMA_20 µM Eosin Y, 7.5% GelMA_20 µM Eosin Y, 7.5% GelMA_50 µM Eosin Y and 10% GelMA_50 µM Eosin Y. 7.5% and 10% GelMA were tested with the same concentration of Eosin Y under the different crosslinking times, and the results showed that there were no significant differences between 10 min and 20 min (p=0.4988, Figure 6B). One possibility is that GelMA can fully crosslink with 10 min light exposure, and the additional light exposure did not alter much of mechanical properties of GelMA. The results from different batches of GelMA showed that there is a good consistency between different batches in terms of relatively stable mechanical properties (Figure 6C).
Figure 6. The results of mechanical properties of GelMA. (A) GelMA stiffness under conditions of 10% GelMA_10 μM Eosin Y, 10% GelMA_20 μM Eosin Y, 7.5% GelMA_20 μM Eosin Y, 7.5% GelMA_50 μM Eosin Y and 10% GelMA_50 μM Eosin Y. The stiffness of 7.5% GelMA_10 μM Eosin Y was significantly larger compared to 7.5% GelMA_50 μM Eosin Y (p=0.0409) and 10% GelMA_50 μM Eosin Y (p=0.0213). (B) No significant difference was indicated in the stiffness of GelMA (50 μM Eosin Y) with different light exposure times (p=0.4988). (C) The stiffness of GelMA from Batch 6 with 20 μM and 50 μM Eosin Y showed significant difference (p=0.0492). No significant differences were shown in other batches. * indicates p < 0.05.

**Compaction of GelMA**

The cells densities of $2.5 \times 10^7$, $5 \times 10^7$, $8 \times 10^7$ cells/mL were tested here. Different densities of cells were encapsulated in 10% GelMA and plated into 96 well plates, and each sample was 2 μL (Figure 7A-B). GelMA compaction was observed, and GelMA with higher cell density tend to cause higher compaction. After day 6, the compaction slowed down and stabilized in the
conditions of 10% GelMA_5e7/mL cells and 10% GelMA_8e7/mL cells (Figure 8A). While in the condition of 10% GelMA_2.5e7/mL cells, gel areas became bigger on Day 10 than before. One possibility is that enlarged gel area after Day 10 was caused by incomplete cell encapsulation, and cells grew outside the gel which the stretched gel will help prevent further compaction. (Figure 7C, Figure 8A). When media was added into each well, some cells were washed out of GelMA, and spread to plates surface, then started to grow. At first, during Day 0 and Day 6, cells inside GelMA started to form tissue and pulled GelMA from inside. However, cells outside GelMA grew and proliferated as well, and pulled GelMA from outside, causing some areas of GelMA become bigger. To prevent the issue, in “dog-bone-shaped” stencil systems, stencils were optimized by incubating in 10% F68 Pluronic prior to the assembly, which can help avoid cell attachment, and therefore, gel compaction process can be evaluated more accurately. I observed that higher Eosin Y concentration caused less compaction compared to lower Eosin Y concentration (Figure 8B-D). 50 μM Eosin Y caused higher GelMA stiffness than 10 μM Eosin Y. Stiffer GelMA caused by higher Eosin Y concentration made it harder for cells to deform. That is why compaction process was relatively slow in the 50 μM Eosin Y condition (Figure 7B-C). I found that when cell density was lower than 5 × 10^7 cells/mL, there is not enough cells to compact from both knob regions in “dog-bone-shaped” stencils, and cells tended to form small “ball-shaped” tissue in knob regions (Figure 9A). However, cells of high densities tended to detach from bottom surface and started to collapse in a short time (Figure 9B). Therefore, I incorporated GelMA hydrogel to improve tissue formation and compaction. I found that even with low density of cells (2.5e7 cells/mL), tissue can form and compact in GelMA hydrogel within “dog-bone-shaped” stencil (Figure 9C). Moreover, cells encapsulated in GelMA can prevent tissue from detachment from the substrates.
Figure 7. Representative images of cells encapsulation. (A) Cells with the density of 2.5e7/mL were encapsulated in 10% GelMA (20 μM Eosin Y) on Day 0. (B) Cells with the density of 5e7/mL were encapsulated in 10% GelMA (20 μM Eosin Y) on Day 0. (C) Cells with the density of 2.5e7/mL were encapsulated in 10% GelMA (20 μM Eosin Y) on Day 10.

Figure 8. Quantification of tissue occupied area (normalized to Day 0). (A) GelMA compaction showed no significant difference between 10% GelMA encapsulating different densities of cells on the same day. The area of 10% GelMA_8e7/mL cells condition on Day 6 was significantly larger than Day 0 ($p=0.0410$). (B) The area Cells encapsulated in GelMA (10 μM Eosin Y) significantly decreased on Day 6 compared to Day 0 ($p=0.0319$). On Day 15, the areas between conditions of 20 and 50 μM Eosin Y indicated significant difference ($p=0.0492$). (C) 10% GelMA compaction indicated the similar trends as 7.5% GelMA encapsulating cells with the same density. (D) The conditions of cells only and 7.5% GelMA_10 μM Eosin Y showed significant difference between Day 0 and Day 6 respectively ($p=0.0143$, $P=0.0349$). On Day 10 and Day 15, the area of
cells only was significantly smaller than those of 7.5% GelMA_10 μM Eosin Y and 10% GelMA_50 μM Eosin Y. * indicates $p < 0.05$.

Figure 9. Representative images of tissue formation in “dog-bone-shaped” stencils on Day 6. (A) Cells with the density of 2.5e7/mL compacted and formed “ball-shaped”. (B) Cells with the density of 8e7/mL detached from the knob regions and started to collapse. (C) Cells with the density of 2.5e7/mL encapsulated in 7.5% GelMA (20 μM Eosin Y) compacted without detachment.
In this study, we optimized the previous developed iPSC-μHM models by modifying surface chemistry during substrates fabrication, and investigated various GelMA conditions for cell encapsulation. In the experiments of surface modifications for substrate fabrication, the results showed that the prolonged dPBS incubation reduced the autofluorescence intensity significantly, and FN bioactivity did not show significant differences, compared to previous substrates without dPBS incubation. Kwahun Lee et al. have found autofluorescence intensity can be influenced by pH (Lee et al., 2013). An environment with high pH can promote the formation of red emitters, but higher pH would suppress the generation of red emitters and accelerate the generation of yellow emitters. Lower pH had the potential to reduce autofluorescence intensity, however, would induce other emitters, such as green and blue emitters. They also added hydroquinone to eliminate autofluorescence, and found hydroquinone incubation can eliminate autofluorescence at a very high pH. In the meantime, the reduction of autofluorescence caused by addition of hydroquinone was strongly related to pH. For example, pH at 7 would excite other fluorescence peaks (Carmichael & Mander, 1967). Overall, adjusting pH or addition of other chemicals are not ideal ways to reduce autofluorescence in our system.

In addition, we investigated the mechanical properties of GelMA for its potential application in our iPSC-μHM model. In previous study, it has been proven that mechanical properties are related to concentrations of photo-initiator. Interestingly, the results in our study showed that there is no significant difference of stiffness between 7.5% GelMA and 10% GelMA with concentrations of Eosin Y from 10-20 μM. One possibility is that the degree of substitution of GelMA is much lower
than we expected, causing less chemical crosslinking of polymer chains (Van et al., 2000). With higher degree of substitution, the mechanical properties would vary significantly by reducing or increasing concentrations of Eosin Y as well as concentrations of GelMA. Based on the protocol that we used and modified, the addition of MA at a constant and slow rate of 0.5 mL/min during synthesis process and frequent water changing during dialysis process helped make sure the batch to batch consistency in terms of mechanical properties, which is important in GelMA application. However, it also led to the lower degree of substitution than expected, which is consistent with the observations by Kerscher et al. (Kerscher et al., 2017) following the similar protocol. Zhu et al. proposed a highly controllability one-pot strategy to ensure the batch to batch reproducibly and controllability when producing GelMA (Zhu et al., 2019). In their study, they successfully exhibited highly consistent GelMA in terms of highly consistent compositional, structural, and functional properties between different batches with expected degree of substitution via the one-pot method. In the compaction study, we compared conditions of cells only and cells encapsulated in GelMA in “dog-bone-shaped” stencils. When tissue in the cells only condition started to collapse, the cells encapsulated in GelMA still continued to compact without any detachment. When GelMA was stiffer, it became harder for cells to compact, which could simulate the stiff tissue condition. It could be a potential way to simulate hardening of heart muscle due to HCM by applying GelMA with different mechanical properties. Based on this, through GelMA encapsulating cells, not only could we simulate tissue stiffness, but also slow down the process of tissue detachment and collapse in “dog-bone-shaped” stencils.
Overall, the study demonstrated an effective way to optimize the iPSC-μHM model by optimizing surface chemistry and explored the potential application of GelMA to optimize the tissue formation in the future. GelMA has shown its promising potential for our iPSC-μHM model to study HCM.
Chapter 5: Limitations and Future Work

From this study, we have demonstrated an effective way to reduce autofluorescence and optimized our previously developed substrate fabrication process, and investigated the possibility of application of GelMA in our iPSC-μHM model. However, there are still some problems to be solved and some limitations to be overcome to optimize our model.

Although the prolonged period of dPBS incubation has showed the successful reduction of autofluorescence during surface modification process of substrates, there are still some autofluorescence shown on the substrates surface, affecting the observation and imaging of micro heart muscle tissue, and capture and analysis of calcium transient. Moreover, 24 hours of dPBS incubation would make the process of substrate fabrication more time-consuming, on the basis of long preparation time, such as PDMS curing process and sterilization process. dPBS incubation is a simple and effective way so far, but a better way still needs to be created. We’ve known that the existence of secondary amine is the key to generate emissive species and cause autofluorescence, a possible way to reduce the autofluorescence is to use alternative silanes besides APTES to minimize secondary amine groups.

C2C12 encapsulation in GelMA has shown a promising application to be used in our iPSC-μHM model. However, the degree of substitution of methacryloyl group remains low following the protocol that we are using, and the relationship between mechanical properties and thickness of GelMA or total volume of GelMA on the basis of different crosslinking time is still not clear. The crosslinking condition and mechanical properties of GelMA that we synthesized are limited by
GelMA concentrations, for example, low concentration of GelMA makes it hard to crosslink with low methacryloyl substitution. Modification on increasing the methacryloyl substitution degree may be needed to increase mechanical tunability of GelMA, and more experiments may need to be conducted to investigate the relationship of crosslinking time and thickness of GelMA and its effects on mechanical properties of GelMA to get more accurate mechanical properties of GelMA used in cells encapsulation.
References

* Papers that the author of this thesis contributed to.


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