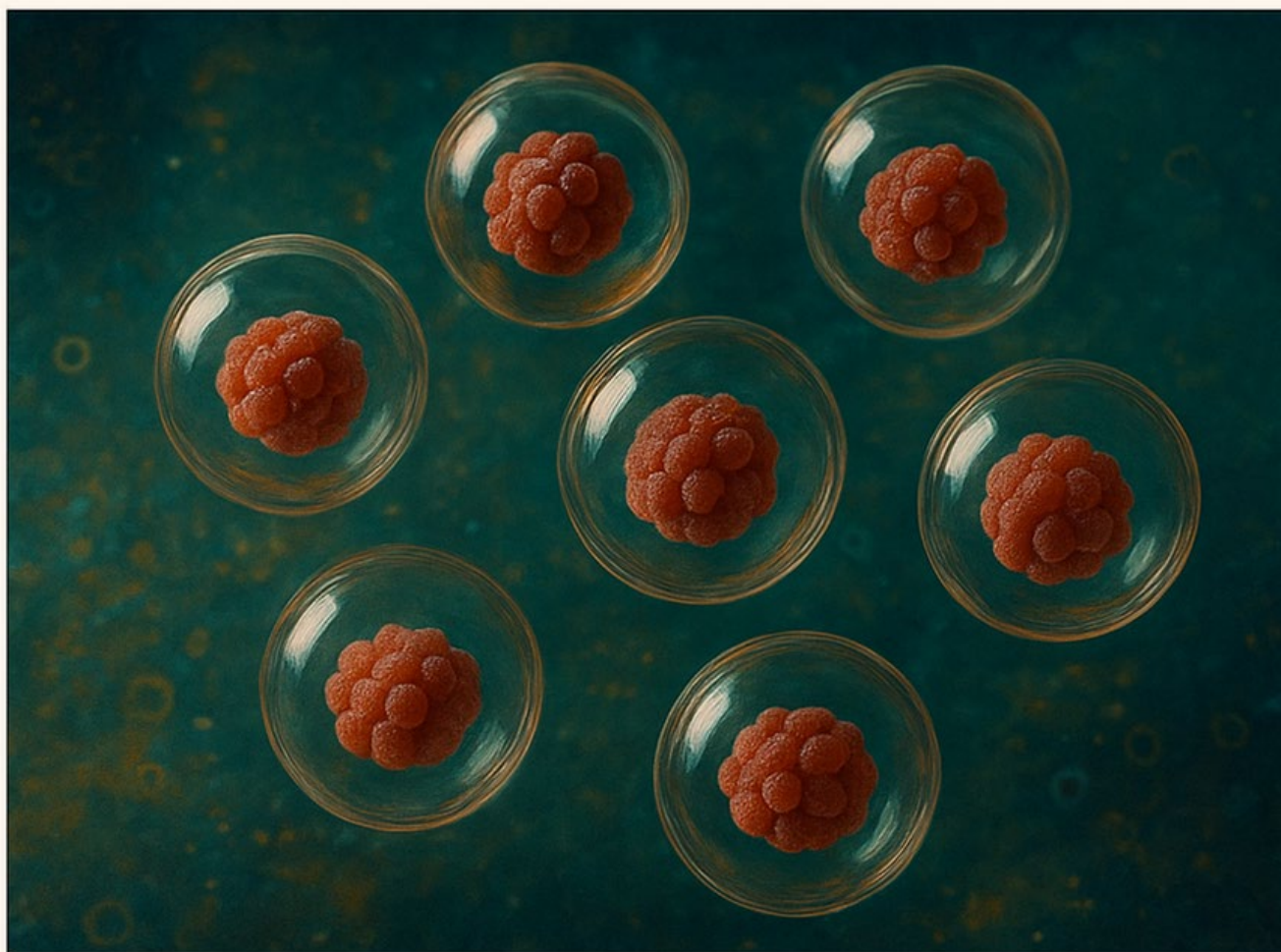


Microfluidics-assisted High-throughput Cell Encapsulation for Microtissue Engineering: from Cancer Models to Artificial Pancreatic Islets



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Doctoral Thesis



Ph.D. thesis prepared within the Warsaw-4-PhD doctoral school at the

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Submitted by

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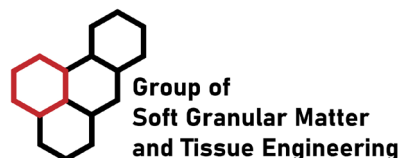
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dr. hab. Jan Guzowski



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Funding sources and collaborations



THESIS LAYOUT

PART-A: High-throughput Encapsulation System Development

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Droplet Microfluidics and High-throughput Microdroplet Generation for Cell Encapsulation

Chapter 2

Crosslinking Conditions Optimization and On-the-fly Crosslinking System Development

Chapter 3

Hydrogel Optimizations for 3D-Cell Culture Application

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Re-encapsulation of Droplets in Droplets for Compartmentalized Co-culturing of Cells

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Abstract

Droplet microfluidics-based technologies for 3D-cell culture and microtissue engineering are receiving increasing interest in the fields of biomedical engineering, biotechnology, and even preclinical drug development. However, balancing throughput of production, droplet monodispersity, and crosslinking efficiency is quite challenging. In the Thesis, I demonstrate an experimental system suitable to produce more than 50000 crosslinked hydrogel (gelatin-based) droplets of $\sim 200\ \mu\text{m}$ diameter within a minute, with 50 cells per droplet on average. Such high droplet production rates have only been used for encapsulating low cell numbers (<10 cells/droplet) or single cells previously, with much smaller droplet diameters. Owing to the high cell number in each droplet, the hydrogel microspheres were suitable for microtissue engineering. Additionally, I also report complex droplet-in-droplet hydrogel structures to allow compartmentalized co-culturing of different cell types. Process parameters such as flow rates, crosslinking light wavelength, irradiance, exposure time, distance from the crosslinking lamp, and temperature during the crosslinking process were optimized.

A two-chip microfluidic system was developed. First, a chip with parallel channels produced the cell-laden microdroplets, which subsequently flowed to the next chip, where they were crosslinked in flow and finally collected. I showed how different chip designs could affect the droplet production and derived the final design to produce highly monodisperse droplets irrespective of high flow rates, with good distribution of cells in each droplet. Next, crosslinking efficiency was demonstrated at different crosslinking light wavelengths and irradiances at a fixed time of UV exposure for on-chip crosslinking. An interesting phenomenon of a well crosslinked droplet core with a partially crosslinked shell was observed in poorly crosslinked droplets at low irradiances. A “sweet spot” was identified in terms of crosslinking conditions to allow production of completely crosslinked droplets without significantly affecting the viability of the encapsulated cells within. For the complex droplet-in-droplet system, the microdroplet “cores” were produced with the optimized conditions and were encapsulated in a second hydrogel layer (“shell”). Similar optimizations of flow rate and crosslinking light intensity, in a stationary condition, were performed to ensure efficient re-encapsulation of droplets.

Different gelatin-based hydrogels were then tested for use in different applications using the two abovementioned systems. I have used the system for disease modelling and cell therapy applications. Firstly, I performed encapsulation and co-encapsulation of leukaemia and bone marrow stroma cells to evaluate drug resistance in the leukaemia microenvironment model. I also demonstrated encapsulation of cancer cells in the “cores” and fibroblasts in the “shell” to use the droplet-in-droplet systems for disease modelling and drug testing. Secondly, pancreatic β -cells were encapsulated in the microdroplets with $>85\%$ viability as cell therapy for Type 1 diabetes. Additionally, mesenchymal stem cells (MSCs) were reprogrammed within porcine pancreatic ECM (ppECM) containing droplets to function as pancreatic islet-like cells. Attempts were made with the droplets-in-droplet system to enable vasculature development in the artificial pancreatic islets. The thesis shows the immense potential of microfluidics in the field of biomedical sciences to design robust systems with high physiological relevance.

Streszczenie

Technologie oparte na mikroprzepływach kroplowych do hodowli komórek 3D i inżynierii mikrotkanek cieszą się rosnącym zainteresowaniem w dziedzinie inżynierii biomedycznej, biotechnologii, a nawet przedklinicznego rozwoju leków. Jednym z głównych wyzwań pozostaje jednak zrównoważenie wydajności produkcji, monodispersyjności kropli oraz efektywności sieciowania. W pracy dyplomowej przedstawiam system eksperymentalny umożliwiający wytwarzanie ponad 50 000 usieciowanych kropli hydrożelu (na bazie żelatyny) o średnicy około 200 μm w ciągu minuty, ze średnią liczbą 50 komórek na kroplę. Tak wysokie tempo produkcji kropli było dotychczas wykorzystywane wyłącznie do enkapsulacji niewielkiej liczby komórek (<10 komórek/kropla) lub pojedynczych komórek, przy znacznie mniejszych średnicach kropli. Dzięki wysokiej liczbie komórek, uzyskane mikrosfery hydrożelowe były odpowiednie do zastosowań w inżynierii mikrotkanek. Ponadto przedstawiam również złożone struktury hydrożelowe typu „kropla w kropli”, które umożliwiają współhodowlę różnych typów komórek w oddzielnych komorach. Zoptymalizowano parametry procesu, takie jak: natężenie przepływu, długość fali światła sieciującego, natężenie promieniowania, czas ekspozycji, odległość od źródła światła i temperatura podczas procesu sieciowania.

Opracowano dwuchipowy system mikroprzepływowy. W pierwszym chipie wyposażonym w równoległe kanały generowane były mikrokrople zawierające komórki, które następnie przepływały do drugiego chipa, gdzie były sieciowane w przepływie i ostatecznie zbierane. W pracy pokazuję, jak różne projekty chipów mogą wpływać na wytwarzanie kropli, także przedstawiam finalny projekt umożliwiający uzyskanie wysoce monodispersyjnych kropli niezależnie od wysokich prędkości przepływu, z równomiernym rozkładem komórek w każdej kropli.

Następnie zbadano skuteczność sieciowania przy różnych długościach fal światła sieciującego i natężeniach promieniowania przy stałym czasie ekspozycji na promieniowanie UV w celu sieciowania na chipie. W przypadku słabo usieciowanych kropli przy niskim natężeniu promieniowania, zaobserwowano interesujące zjawisko dobrze usieciowanego rdzenia kropli z częściowo usieciowaną powłoką. Zidentyfikowano „punkt optymalny” pod względem warunków usieciowania, który pozwala na wytwarzanie całkowicie usieciowanych kropli bez znaczącego wpływu na żywotność zamkniętych w nich komórek. W przypadku złożonego systemu „kropla w kropli”, „rdzenie” mikrokropli zostały wyprodukowane w zoptymalizowanych warunkach, a następnie zamknięte w drugiej warstwie hydrożelu („powłoce”). Przeprowadzono analogiczną optymalizację parametrów przepływu oraz intensywności światła w warunkach stacjonarnych w celu zapewnienia skutecznego ponownego zamknięcia kropli.

W dalszej części pracy przetestowano różne hydrożele na bazie żelatyny, wykorzystując oba opisane systemy, w kontekście ich zastosowania w modelowaniu chorób oraz terapii komórkowej. Przeprowadziłam enkapsulację i koenkapsulację komórek białaczkowych i komórek zrębu szpiku kostnego w celu oceny oporności na leki w modelu mikrośrodowiska białaczki. Ponadto zaprezentowałam również enkapsulację komórek nowotworowych w „rdzeniach” i fibroblastów w „powłoce” w celu wykorzystania systemów „kropla w kropli” do modelowania chorób i testowania leków. W kolejnym etapie

przeprowadzono enkapsulację komórek β -trzustki uzyskując żywotność >85%, co wskazuje na możliwość ich zastosowania w terapii komórkowej cukrzycy typu 1. Dodatkowo, mezenchymalne komórki macierzyste (MSC) zostały przeprogramowane wewnątrz kropel zawierających macierz zewnątrzkomórkową pochodzącą z trzustki świni (ppECM), tak aby pełniły funkcję komórek przypominających komórki wysp trzustkowych. Podjęto próby wykorzystania systemu „kropla w kropli” w celu inicjowania rozwoju naczyń krwionośnych w sztucznych wyspach trzustkowych.

Praca ta pokazuje ogromny potencjał technologii mikrofluidycznych w biomedycynie – w szczególności w tworzeniu zaawansowanych, fizjologicznie istotnych systemów do badań przedklinicznych, modelowania chorób oraz rozwoju terapii komórkowych.

Preface

“আমাদের সমুদয় শিক্ষা-দীক্ষা কেবল মনুষ্যত্বলাভের উদ্দেশ্যে মাত্র।”

(“All our education and learning should ultimately serve the cause of humanity.”)

- Jagadish Chandra Bose,

*[Scientist credited with the discovery of life
in plants and inventing the Crescograph]*

Since the beginning of human life, Science has been a companion in progressing humanity. From the first spark generated by rubbing two stones together to development of the wheel, from the invention of machines to the modern day mobile phones, Science and Humanity have come a long way. Science has played a great role in understanding and deciphering mechanisms to use elements from the physical, chemical and biological world around us.

The quest for knowledge has driven scientists towards going deeper and deeper into objects and beings. The extent of this discovery has reached beyond the atomic level into sub-atomic and elementary particles such as quarks, photons, neutrinos, and the earlier discovered electrons. These discoveries showed that it was possible to manipulate particles at micro and nano-levels to broaden their applications, leading to the emergence of the now popular fields of micro and nanotechnology.

The concept of “miniaturization” to make mechanical, electronic, or biological systems smaller can be dated back to the use of very small objects used in ancient craftsmanship for jewellery and clock-making. Miniaturization further received a boost as the visualization of objects beyond the limits of the human eye could be achieved through microscopes. The first microscope was developed by Dutch spectacle makers, Hans and Zacharias Janssen, in 1590, which was refined in the 17th century by Antonie van Leeuwenhoek, enabling the observation of bacteria, sperm cells, and red blood cells, effectively initiating the age of microscale science.

The modern era of miniaturization truly accelerated in the 20th century with the development of microelectronics. The invention of the transistor in 1947 by John Bardeen, William Shockley, and Walter Brattain at Bell Labs marked a crucial turning point. This breakthrough enabled electronic components to be drastically reduced in size, leading to the integrated circuit (IC) in the 1950s and later, the microprocessor in the 1970s. These developments have not only revolutionized electronics but have also contributed to the field of biomedical sciences, making diagnostics faster and accurate. Today, miniaturization has become a part of biological, chemical, and physical sciences to develop innovative equipment, engineer improved systems for better process control and higher production rates, and also speed up testing and optimization to achieve faster and more precise solutions.

This thesis aims at carrying forward the work in the field of miniaturization through the existing field of microfluidics to develop miniaturized models to study life-threatening diseases such as cancer and also as a cell therapy-based treatment for a disease like Type-I-diabetes with limited treatment options. The work done in this thesis is expected to contribute to the field of biomedical sciences through the integration of microengineering.

PART-A: High- throughput Encapsulation System Development

Chapter 5 : Droplet Microfluidics and the Process of High-throughput Monodisperse Droplet Generation for Cell Encapsulation

Microfluidics has been a greatly popular field of research over the last two decades. It deals with manipulation of very small volumes of fluids (even down to femtoliters) using engineered devices (known as “chips”) in a controlled manner. The chips facilitate high precision and reproducibility. Till date, microfluidics has been used for various high-throughput applications like lab-on-chip and has led to emergence of subfields like droplet microfluidics. In this chapter, first, some key concepts of microfluidics will be explained, followed by focused explanation of the different strategies of droplet microfluidics.

History of Droplet Microfluidics

The first monodisperse microfluidic droplet generation process was demonstrated by Thorsen and colleagues in 2001¹. They made water-in -oil emulsion droplets with different oils (variants of decane) with different surfactant (Span 80) concentrations using a device similar to a T-junction design. Around the same time, an interfacial tension-driven technique for producing highly monodisperse emulsion droplets ($CV < 5\%$) was developed by Sugiura and colleagues². The process design is currently known as step-emulsification and several chip geometries have evolved over the years to further improve droplet monodispersity.

The Basics of the Process

Droplet microfluidics is a branch of microfluidics dealing with the production of droplets with diameters in the micrometer range and discrete volumes using immiscible fluids with the help of a microfluidic chip. Fluid behaviour differs at the macroscale and microscale. In particular, at the microscale the effects associated with interfacial tension, mass and heat transfer, and viscous forces become dominant³. The In particular, at the microscale, the effects associated with interfacial tension, mass and heat transfer, and viscous forces become dominant³. The liquid phase used to form the droplets is conventionally referred to as the “dispersed phase,” while the external phase in which the droplets are created and carried is referred to as the “continuous phase”. Generally, in droplet microfluidics, the continuous phase needs a surfactant to minimize interactions at the droplet-droplet interfaces and prevent coalescence of the generated droplets. Droplet formation can be achieved by any of the two methods³: Droplet formation can be achieved by any of the two methods³:

- Spontaneous breakup of a liquid stream driven due to hydrodynamic pressure differences (passive droplet generation)
- Breakup forced by external actuators (active droplet generation)

Most common, two-phase systems include water-in-oil (W/O) and oil-in-water (O/W) emulsions, while more complex droplet structures such as water-in-oil-in-water droplets can also be generated. In this project, W/O droplets were generated and used for various cell culture applications initially. In the later phase of the project, these droplets were further re-

encapsulated in a larger second droplet by a hydrogel layer to form water-in-water-in oil (W/W/O) -like double emulsion droplet generation process to act as 3D culture systems for multi-cell types in segregation (demonstrated in Chapters 4, 5, and 7).

Common Microfluidic Channel Geometries

For most applications, uniformity in droplet size or, in other words, monodispersity of an emulsion, is a crucial factor. For most applications, uniformity in droplet size or, in other words, monodispersity of an emulsion, is a crucial factor. The channel designs mostly used for monodisperse droplet production have been discussed below⁴.

1. **T-junction**: Probably the simplest and most commonly used channel design, the T-junction, is designed like the letter ‘T’. One of the ends of the horizontal “head” is the inlet for the continuous phase, while the end of the vertical “body” is the inlet for the dispersed phase. As the dispersed phase meets the continuous phase at the junction, the pressure building up at the interface pushes the dispersed phase downstream into the outlet channel. As the interface is shifted downstream, the neck of the dispersed phase thins and eventually breaks, resulting in the formation of a droplet, which is then carried downstream by the continuous phase (**Figure 1-1.A**).
2. **Flow-focusing**: One of the earliest designs used in droplet microfluidics, the flow-focusing junction has a tapering-mouthed channel in the centre, with two equal-sized channels flanking on either side. The interior channel contains the dispersed phase, while the side channels deliver the continuous phase. As the continuous phase reaches the interior channel, it squeezes the dispersed phase from both sides, resulting in the formation of even-sized droplets (**Figure 1-1.B**).
3. **Co-flow**: This geometry can be somewhat considered a variant of the flow-focusing geometry. Essentially, it is a 3D design with a smaller capillary for the dispersed phase flow in a larger capillary for the continuous phase flow. As the dispersed phase enters the immiscible continuous phase, it is broken off into smaller droplets due to shear stress. The co-flow geometry is advantageous as it can prevent the involvement of the boundary from affecting droplet sizes. Also, compared to the flow-focusing technique, flow imbalances from the opposite channels leading to polydisperse droplets can be avoided as a unidirectional flow of the continuous phase happens in this geometry (**Figure 1-1.C**).
4. **Step-junction**: This design is one of the most robust methods for generating highly monodisperse droplets. In this design, the dispersed phase flows through a straight channel with a widened mouth (rectangular, wedge, or triangular-shaped). The continuous phase flows through a deeper channel next to the mouth of the dispersed phase channel. As the dispersed phase forms a growing spherical ‘bulb’ (thanks to the available space), the Laplace pressure inside the bulb gradually decreases, eventually resulting in droplet pinch off (**Figure 1-1.D**). Unlike the T-junction and flow-focusing designs, flow rate variations do not affect the droplet sizes in this design significantly.

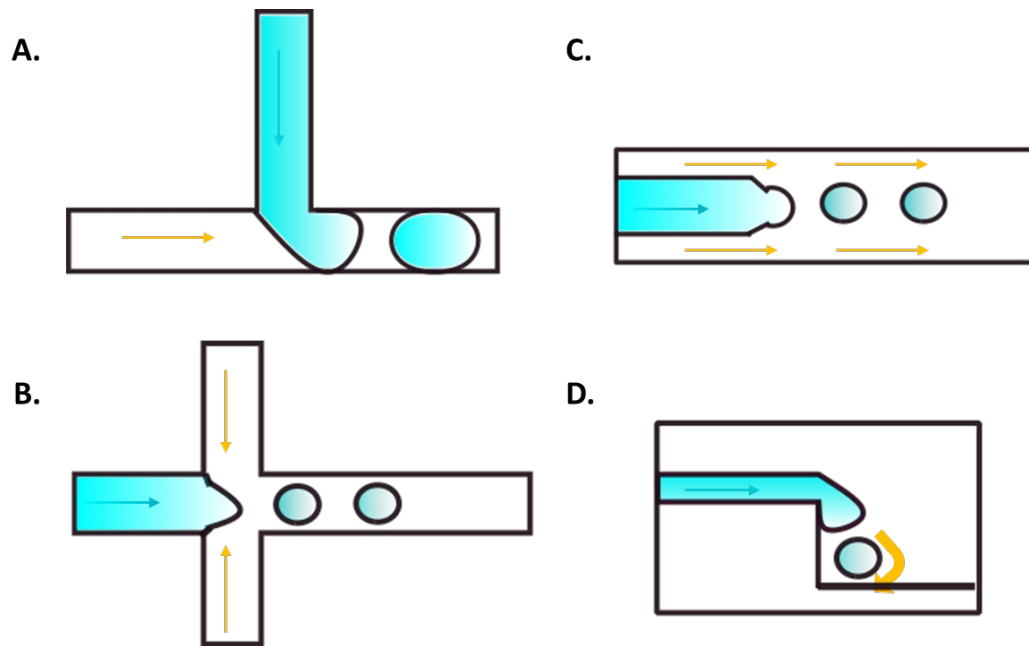


Figure 1-1: Common microfluidic channel types. Droplet formation by the interaction of the dispersed phase (flow direction shown by blue arrows) and the continuous phase (flow direction shown by yellow arrows) in **A.** T-junction, **B.** Flow-focusing, **C.** Co-flow, and **D.** Step-junction microfluidic device.

Droplet sizes can be determined by factors such as the following:

- Viscosity of the immiscible phases
- Surfactant type and concentration
- Channel treatment (hydrophobic or hydrophilic)
- Nozzle orifice dimensions
- Flow rates (only for T-junction and flow-focusing designs)

The above-mentioned devices are based on pressure differences and are passive methods. Another type of device is based on electrohydrodynamic (EHD) forces, which include dielectrophoresis (DEP)-driven systems, which enable droplet generation by the flow of polarizable fluids towards a higher electric field. Another EHD-based design is electrowetting on dielectric (EWOD), where electric fields are used to increase channel hydrophilicity to allow lower contact angles.

The fluidic components

In W/O droplet systems, commonly water or similar fluids are used for droplet generation, but hydrogels are also very popular materials for droplet generation. The hydrogels are liquid initially, but based on exposure to factors like temperature, pH, light, or chemicals, these can form stable gels by crosslinking available functional groups. Droplets can be generated with the hydrogels and then transferred to a water-like external phase after crosslinking. Such hydrogels are very good materials for 3D cell culture applications. Hydrogels have been described in more detail in one of the following chapters (Chapter 3). It is also possible to generate microdroplets using gas as the dispersed phase in microbubble generating systems, where the same channel designs can be used as described above.

The continuous phase in W/O systems comprises the oil with the surfactant to allow droplet formation and to prevent coalescence. The surfactants (surface active agents) are amphiphilic molecules that interact with both of the immiscible phases and lead to a decrease in surface tension at the interface of the two fluids. The Gibbs adsorption isotherm for dilute solutions can be used to estimate this decrease in surface tension using the formula⁵:

$d\gamma = -\sum C_i \cdot d\mu_i$ where, $d\gamma$ is the change in surface tension, C_i is the increased interfacial concentration, and μ_i is the chemical potential of the surface active agent.

For our applications, a fluorinated oil, HFE-7500 (commercial name: Novec® 7500), was used in combination with the fluoro-surfactant, PFPE-PEG-PFPE. Structurally, this perfluoro polyether (PFPE) surfactant is classified as a triblock copolymer with a centrally located polyethylene glycol (PEG) block flanked on either side by two PFPE blocks⁶. It is formed by the combination of a PEG diamine and the acid chloride of Krytox⁷. This acid chloride part reacts with water in the dispersed phase and produces surface-active salts to remain dispersed between the fluoro-oil and the aqueous phase boundary. This helps in maintaining the structural integrity of the droplets.

Dynamics of droplet formation

Since the system involves two immiscible liquids, an interface is formed between them, and manipulation of this interface helps in forming the droplet. The interface contributes to the free energy of the system and is proportional to the surface area of the interface⁸. So the force required to produce the droplet by altering the interface can be given by:

$F = \frac{dE_\gamma}{dr} = \frac{\gamma dA}{dr}$ where, F is the interfacial force, E_γ is the free energy at the interface, dr is the displacement at the interface, γ is coefficient of interfacial tension and A is the surface area of the interface.

The pressure exerted by the curved interfaces (P_γ) of the droplet is known as Laplace pressure, and it can be calculated for a droplet with a surface area of $4\pi r^2$ as:

$P_\gamma = \frac{F}{A} = \frac{1}{A} \gamma \frac{dA}{dr} = 2\gamma \cdot \frac{1}{r}$ where r is the radius of the droplet.

Considering an arbitrary surface, the concave and convex curvatures produce opposing pressures, and their sum is proportional to the Laplace pressure. The orientation of this pressure is towards the concave interface, and hence a deformed droplet restores its spherical shape in the immiscible continuous phase. The cylindrical morphology of the dispersed phase is not favoured in the continuous phase, leading to the formation of an elongated neck, and is driven by the Laplace pressure breaking it into spherical droplets. This phenomenon is termed the Rayleigh-Plateau instability.

For T-junction devices, the droplet formation process is flow rate dependent. Predominantly, the droplet formation process in such systems is determined by the parameter capillary number (C_a) to determine how and when the dispersed phase breaks into droplets⁹. The C_a can be determined using the formula:

$C_a = \eta v / \sigma$, where η is the viscosity of the continuous phase, v is the velocity of the continuous phase, σ is the interfacial tension between the two immiscible phases.

At low C_a values, the droplet formation follows a squeezing regime, at intermediate flow rates it follows a dripping regime, and transitions into jetting at higher C_a values (**Figure 1-2.A.**). When the dispersed phase enters the cross-section at which it meets with the continuous phase, it is separated from the boundaries of the channel by films of the continuous phase on either side and is dragged forward¹⁰. The interfacial forces allow the cylinder formation, where the pressure drops as the droplet head moves forward and is forced to break off due to the shear forces of the continuous phase flow.

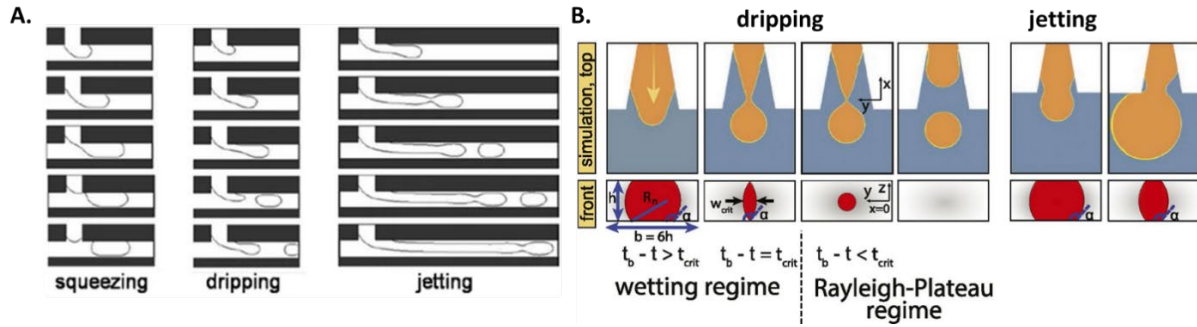


Figure 1-2: Droplet formation methods. A. Droplet formation in a T-junction device at different flow regimes. B. Droplet formation in a step-emulsification device. (Images from ref. 5 and 8).

In the case of a step emulsification device, as the dispersed phase flows into the deeper channel (reservoir) through the shallow wedge-shaped mouth, it meets the continuous phase¹¹. In the wedged channel, the dispersed phase forms a tongue-like structure (**Figure 1-2.B.**). As this proceeds into the deeper reservoir (should be bigger than the droplet diameter expected), the dispersed phase head starts expanding, forming a spherical bulb in the continuous phase reservoir. The bulb remains connected to the main body of the dispersed flow in the wedge, forming a thin neck that gets thinner as the bulb grows, which is driven by Laplace pressure. At a point due to shear stress, the neck breaks off (pinch-off), forming a droplet due to Rayleigh-Plateau instability. The Laplace pressure difference between the neck and the bulb is dependent on the curvatures of the two phases. The curvature of the neck (κ_{Neck}) can be given by the formula:

$$\kappa_{Neck} = \frac{1}{R_{Neck}} = \frac{-2 \cos(\alpha)}{h}$$

where, R_{Neck} is the radius of curvature of the neck, α is the contact angle formed by the dispersed phase as it proceeds into the reservoir from the wedge, h is the channel height.

For a step emulsification device, the uniform droplet formation happens in the dripping regime where pinch-off occurs in a regular manner. For very high flow rates, the jetting regime can be triggered, leading to irregular pinch-off resulting in polydispersity in droplet diameters. The inertial and viscous forces can also affect pinch-off.

This chapter deals with the initial optimizations to develop the system for producing cell encapsulating droplets in high throughput using the photocrosslinkable hydrogel, Gelatin methacryloyl (GelMA), using microfluidic chips with T-junction and step emulsification designs. Firstly, durable chips with the required design to obtain highly monodisperse droplets at fast production rates were optimized. Various flow rates and volume fractions of the continuous phase to hydrogel phase were tested to obtain the highest monodispersity in the droplets. The finalized chip design demonstrated a production rate of upto 10^5 droplets per

minute with monodispersity with a coefficient of variance <3% with an average of 50 cells encapsulated per droplet at a cell concentration of up to 20 million/mL.

1 Materials and Methods

1.1 Microfluidic chip fabrication

Two chip types were commonly used for microfluidic droplet generation during the project: polycarbonate chips and Polydimethylsiloxane (PDMS)-glass chips.

1.1.1 Polycarbonate chip fabrication

For polycarbonate chips, the chip design was micromilled onto the polycarbonate sheet. Another flat, unmilled polycarbonate sheet was used to close the channels. Both parts had holes in the corners in the perfect alignment to insert small steel needles to hold the chip and the cover together. The assembled chip was placed inside the two heating blocks in a hot press and tightly (not forced) screwed to hold the chip in place. The temperature of the hot press was set to 130°C, and the air pressure was set according to the chip's total surface area (0.1 Pa/cm²). The start button was pressed, and the process was allowed to complete for 25 minutes. After 25 minutes, the stop (pressure release) button was pressed, and the temperatures were set to minimum. At around 40-50°C, the heat blocks were unscrewed, and the bonded chip was removed. After the chip was completely cooled to room temperature, Novec 1720 was flowed through the channels, and the chip was left in a drying chamber at 75°C for 10 minutes. After being removed from the drying chamber, the chip was ready to use.

1.1.2 PDMS chip fabrication

For the PDMS chip, the channel design was micromilled on a PMMA sheet and cut out. The milled PMMA chip was used to make a negative or master mould. The PMMA chip was fixed at the centre of a petri dish with the channel (milled) side facing up. PDMS pre-polymer and curing agent were mixed thoroughly in a 9:1 ratio and degassed in a desiccator with a vacuum pump. After degassing, the PDMS mixture was poured into the petri dish with the PMMA chip and kept overnight in the drying oven. After taking it out of the dryer, the cured PDMS negative was carefully removed. This negative was treated with trichloro(1H,1H,2H,2H-perfluorooctyl) silane (PFOCTS; Gelest Inc., United States) for 4 hours at 10 mbar pressure in a dessicator. Post-silanization, a new PDMS solution was poured into the PDMS master mould and kept in the drying oven at 75°C for 3 hours. The polymerized chip was carefully peeled off the negative mould, and the inlets and outlets were punched in the designated areas of the chip using a biopsy punch (1 mm diameter).

For bonding the chip to glass, the PDMS chip and glass slide to be used as the channel cover were washed with soap and water, washed with distilled water, and then dried using an air gun. After drying, the chip was placed with the channel side facing up, with the glass slide beside it in a plasma cleaning chamber. The chamber was pressurized to less than 0.4 mbar,

and then the plasma generator was started at high power. Depending on the size of the chip, the exposure to plasma treatment was varied (1-1.5 min). After the treatment was completed, the chamber was aerated to normal pressure, and the sides of the glass and PDMS chip facing upwards were sealed together. The bonded chip was placed on a heating plate for 1-2 hours to remove the water molecules generated during the plasma cleaning process. The chip was removed from the hot plate, cooled down to room temperature, and then the channels were treated with Novec 1720 and dried in the oven at 75°C for 10 minutes.

1.2 Chip Analysis

To observe the milling precision for the channels, the PMMA or polycarbonate chips were checked under an optical profilometer (Contour GT-G, Bruker, Massachusetts, United States) based on white light interferometry (Figure 1C). The profilometer was controlled by Vision 64 software for image acquisition and surface analysis. The acquired images could also be analysed using the open-access software Gwyddion.

1.3 Hydrogel Preparation for Droplets

For droplet production, 6% GelMA w/v was used (GelMA synthesis procedure mentioned in Chapter 2). To prepare the GelMA solution, freeze-dried GelMA was weighed to get 0.06 g. To this, 1 mL of PBS was added, and the solution was stirred at 50°C for 1 hour to dissolve the GelMA completely. The final solution was filtered using a 0.22 µm syringe filter and used for droplet production.

1.4 Droplet Production Process

In general, for droplet production, oil-surfactant was loaded on a 1 mL syringe (Beckton-Dickinson plastic) and connected to the inlet of the droplet production chip (DPC) with a polyethylene tubing (Instech Laboratories Inc., USA). The hydrogel with or without cells (cell maintenance and with cell droplet preparation described in Chapter 2 methods) was loaded in this tubing to avoid cell settlement issues. In a 5 mL syringe, oil (HFE 7500) with 2% fluorosurfactant (PFPE-PEG-PFPE) was loaded into a connected tube to the inlet for the continuous phase of the DPC using a tubing. The two syringes were mounted on the pump (Harvard), and the required flow rates were adjusted, and the process of droplet generation was performed. Generated droplets were collected in an Eppendorf tube. The schematic representation of the droplet generation process can be seen in **Figure 1-3** below.

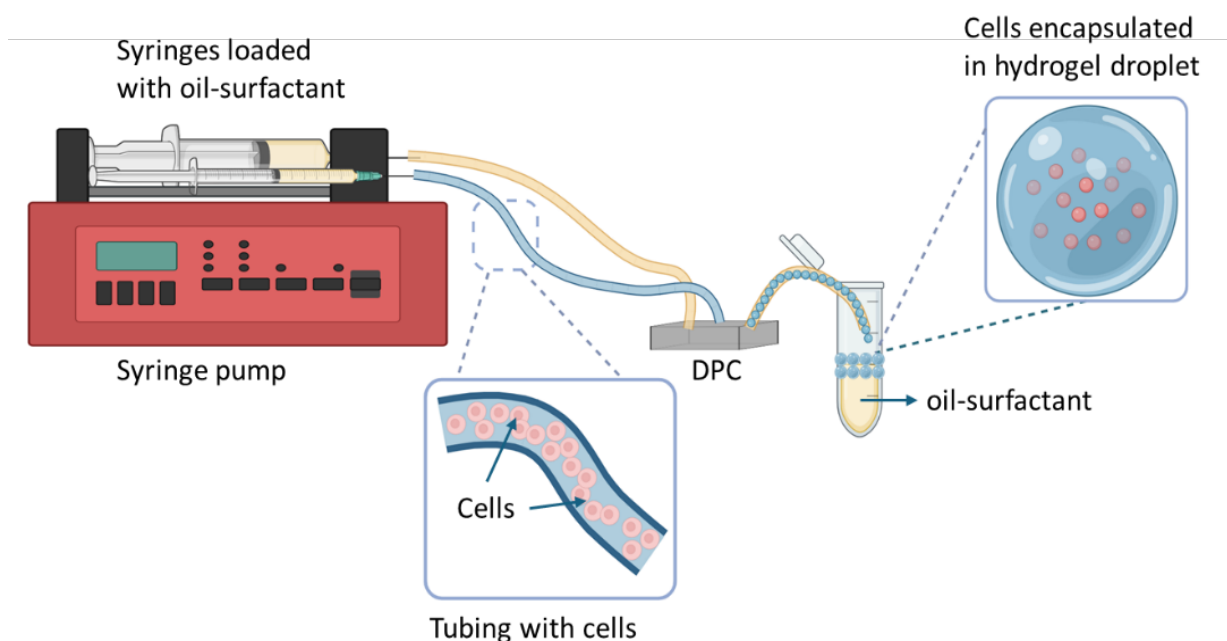


Figure 1-3: Schematic of the droplet production process (Created in <https://BioRender.com>).

1.5 Droplet analysis

The droplet generation process was monitored using a stereomicroscope fitted with a uEye camera and its respective software. Post-production, the droplets were observed under a brightfield, inverted microscope (Nikon Eclipse AS1), and image acquisition and analysis were performed using the NIS-Elements software.

1.6 Graphical and Statistical Analysis

The graphical data representation was made using the Prism 5 (GraphPad) software, along with statistical analysis performed based on the Student's T-test for significance analysis of data. MATLAB programming software was used for histograms.

2 Results and Inferences

2.1 Chip design optimizations

2.1.1 T-junction chip for high throughput

The first attempts were made at a parallel droplet generation chip with a polycarbonate chip with 10 T-junction channels. The chip was fabricated in three layers (**Figure 1-4.A**), where the first layer had a 3 mm height with the inlets for hydrogel and oil-surfactant (marked as “hydrogel inlet” and “oil inlet” respectively) milled along with the distribution system. The second layer (0.75 mm) had 20 inlets- 10 to carry the hydrogel and 10 to carry the continuous phase to the third layer (5 mm) with the T-junction (channel dimensions $180 \times 180 \mu\text{m}$) and

droplet collection channels (triangulated region). Even though monodisperse droplets could be obtained, the GelMA got stuck to the walls of the distribution channels very often, leading to clogging of chips, thereby affecting throughput. The solution to this problem was to clean the chip with hot water (70-80 °C) after detaching the 3 layers. Manufacturing a new chip needed micro-milling every time, making mass production of the chip a time and labor-intensive process.

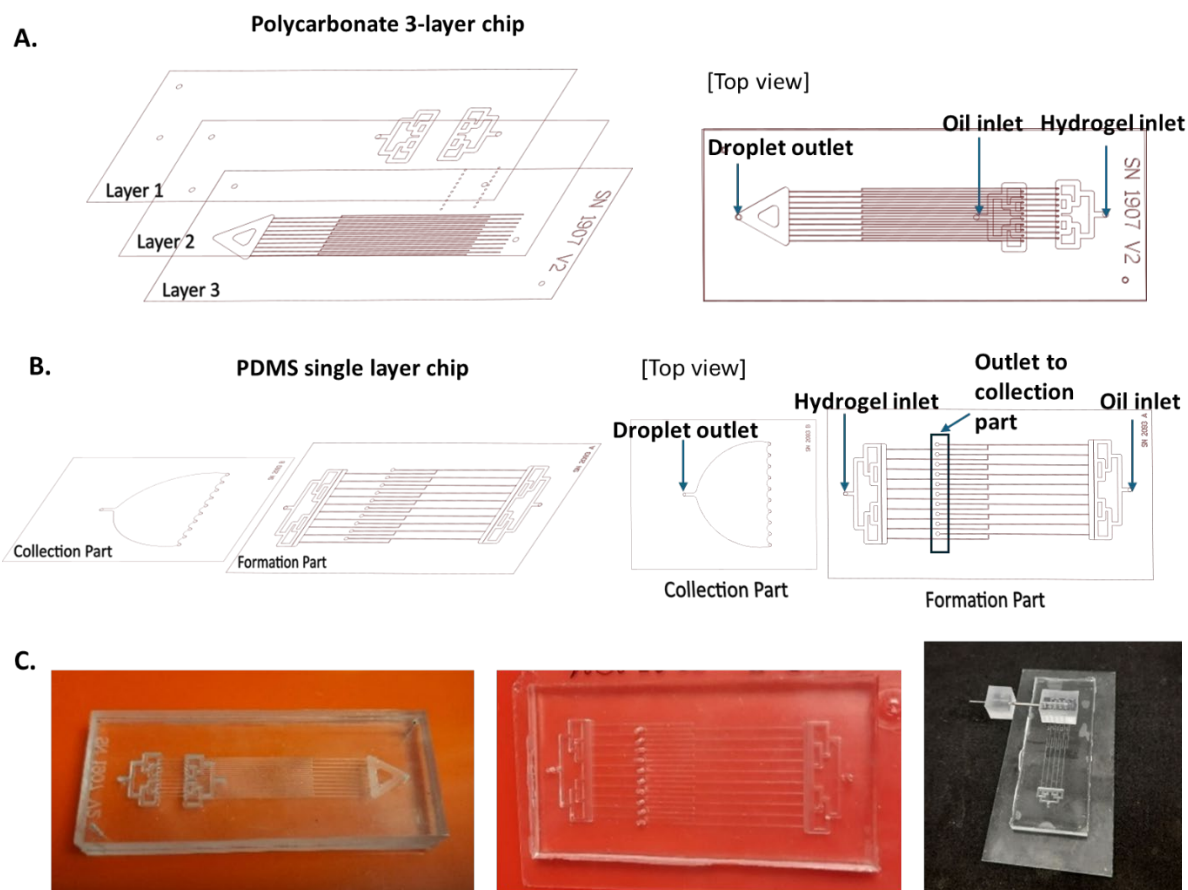


Figure 1-4: T-junction parallel chip designs. **A.** Polycarbonate 3-layer chip and details top view showing details of channel function, **B.** PDMS single layer parallel channel and details of chip in top view. **C.** Images of the fabricated chips.

To avoid complications as stated above, the material for chip production was replaced by PDMS. From a single milled chip on PMMA, hundreds of copies of the required chip could be produced with simple handling steps (detailed procedure described in the methods section above). The 3-layer design was converted into a single-layer, 2-part design (**Figure 1-4.B**); the “formation” part with the hydrogel and oil inlets with 10 T-junction channels leading to 10 outlets for produced droplets, while the “collection part” was a triangular 0.8 mm channel with 10 inlets. The droplet generation process produced highly monodisperse droplets at high throughput. However, the 10 outlets leading to the collection chamber with 10 tubing connectors made the system complicated, as disconnections were common due to inadequate tubing flexibility throughout the generation process. Also, at the collection chamber, residual droplets were common due to irregularities in flow within the chamber.

The 10-channel chip was converted into a 5-channel chip with the same dimensions to avoid complicated assembly. A polycarbonate connector with 5 inlets leading to a single

channel was fitted with flattened 0.8 mm steel needles and connected at the droplet outlets on the PDMS chip (**Figure 1-4.C**). The droplet generation process produced highly monodisperse droplets with slightly reduced throughput compared to previous designs, but the production process was easier to control. However, repeated usage of the chip resulted in breaks at the PDMS outlets or loosening of the needles at the polycarbonate connector inlets (needles need to be cleaned to remove residual/solidified GelMA after every experimental run), leading to leakages resulting in flow rate errors and droplet coalescence in the collector channel.

2.1.2 Circular chip evolution

The parallel channel T-junction chips could not eventually produce consistent droplets and hence were replaced by a step emulsification-based device. The new design was circular in shape with 64-wedge shaped (40 μm depth) step emulsification-based droplet generators located radially (**Figure 1-5**). The hydrogel inlet had 16 channels of 150 μm depth for hydrogel distribution into the wedged channels. Surrounding the 64 channels at a 200 μm depth, the oil-surfactant channel was placed. As the hydrogel fell into the oil-surfactant from the wedge-shaped channels, they broke into droplets at a point (labelled as “pinch-off point”; **Figure 1-5.A** inset). The generated droplets were carried by the oil flow out of the chip.

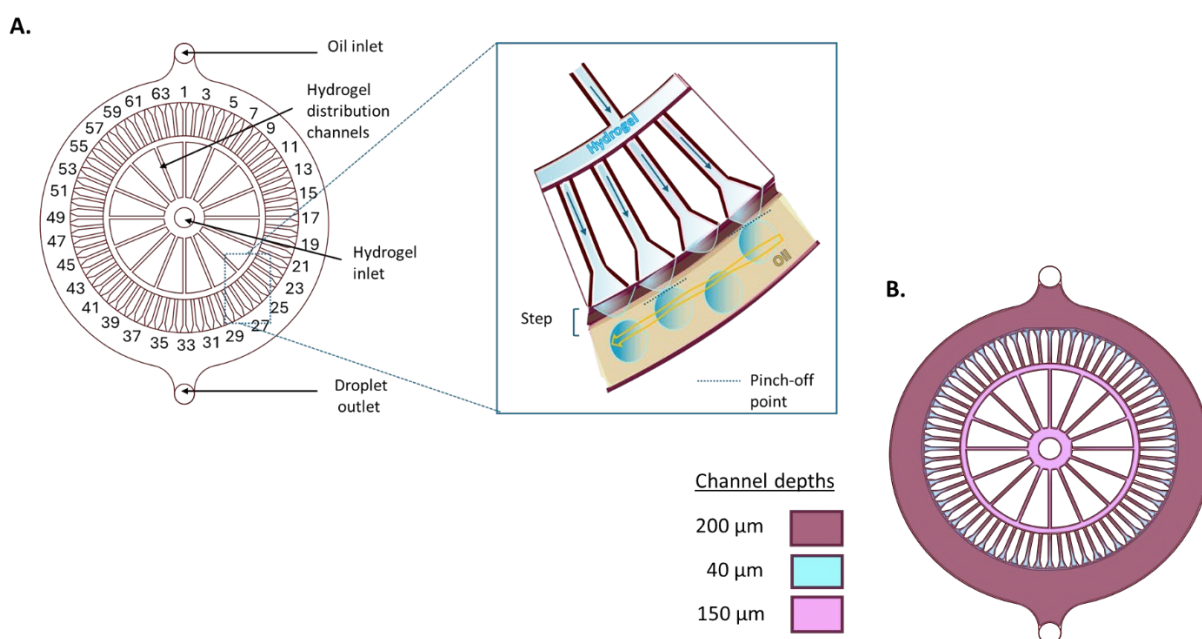


Figure 1-5: The circular step emulsification chip. **A.** Schematic of the chip (every alternate channel number labelled) with the droplet generation process shown in the inset. **B.** Colour-coded representation of the channel depths of the final chip design.

The first design that was tested had a symmetric circular structure with equal widths at the oil inlet and outlet (**Figure 1-6.A**). It produced monodisperse microdroplets quite rapidly (depending on flow rates used) and in large amounts due to the large number of parallel channels. At the outlet, however, a lot of droplet crowding was observed, leading to the coalescence of droplets as they flowed out of the outlet, leading to polydispersity. To avoid this problem, an external oil channel was added to the design to add oil volume and flow to the system (**Figure 1-6.B**). The design prevented droplet coalescence, but the flow rates slowed down in this channel and leading to accumulation and stagnation of droplets in this oil channel.

To substitute the external oil channel, a 5 times deeper oil channel (compared to the 200 μm in the original design) was designed to serve a similar function as the external oil channel (**Figure 1-6.C**). But in this design, the droplets started stacking above each other, forming layers and stagnation of flow at the lowest layers. Finally, the design with the original depth but asymmetrical width of the inlet and outlet area solved all issues with droplet crowding and coalescence.

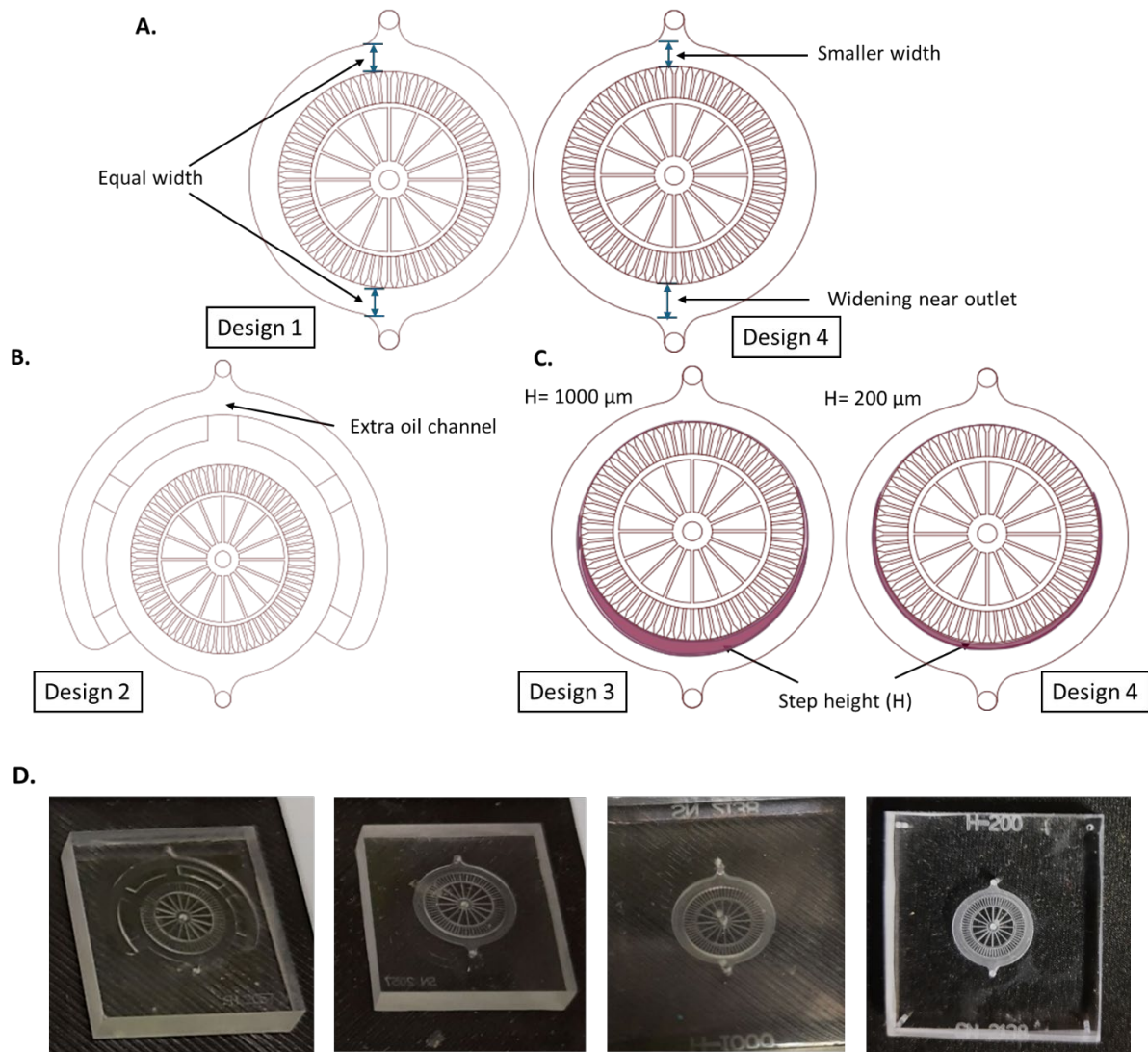


Figure 1-6: Circular chip design evolution. **A.** The first chip design with equal channel widths at the inlet and outlet, compared to the final design (Design 4), with a wider width near the outlet. **B.** The second design with an extra oil channel for added continuous phase flow to prevent coalescence. **C.** The third design was made to facilitate greater continuous phase flow by having a 1 mm deep oil channel (without an added channel) compared to the final design with a 200 μm deep oil channel. **D.** Images of the 4 circular chip designs attempted.

A profilometric analysis of the channels was performed to check for variations in channel dimensions due to precision issues of the micromilling process. The measured channel profiles showed that the average depths of the distribution channels, the wedged channels, and the oil channels were $152.47 (\pm 10.41)$, $49.01 (\pm 7.47)$, and $230.93 (\pm 9.19)$, respectively, differing from the original dimensions slightly (**Figure 1-7A, B** and **Figure 1-5B**). On focusing particularly on the droplet generators, the wedge height (H_w , the height of the top of the

wedged channels from its bottom) varied from 37.2 to 50.6 μm , and the step height (H_s , height from the bottom of the wedge to the bottom of the oil channel) ranged from 170.03 to 194.3 μm (Figure 5C).

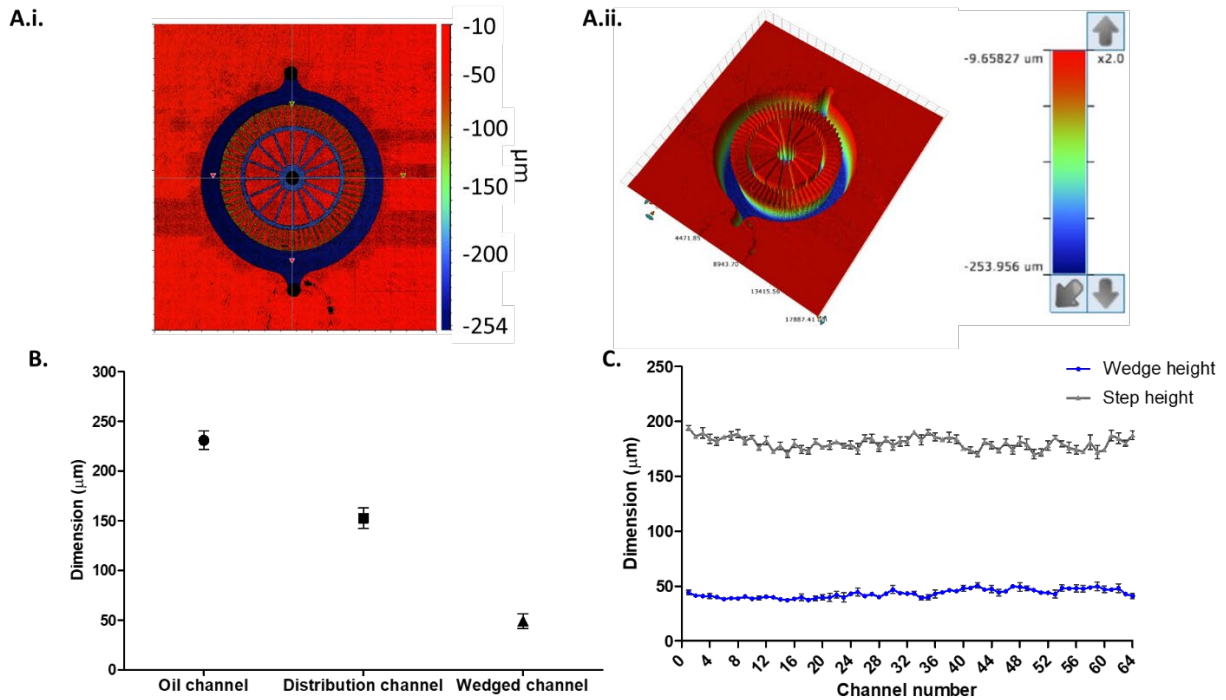


Figure 1-7: Profilometry analysis of the circular chip. **A.** The i. 2D profile and ii. 3D profile of the chip. **B.** Analysis of the different channel depths based on multiple point measurements with respect to the chip's top surface. **C.** The wedge and step heights for each of the 64 channels were measured from 3 milled PMMA chips of the final design.

These milling inconsistencies could lead to slight variations in droplet diameters, which will be dealt with in the next section. This problem can be managed by levelling the chip after milling. For the small wedge areas, spraying with octanol fumes could help remove dust and irregularities from the micromilling process. To summarise, the pros and cons of all the tested chip designs can be found in **Table 1-1**.

Table 1-1: Summary of the tested chip designs based on their design, materials, advantages and disadvantages.

Chip Name	Type	Material	Advantages	Disadvantages
3-layer chip	T-junction	Polycarbonate	- Sturdy chip - Easy to handle	- Micromilling required for fabrication - Hydrogel solidification in channels/ difficult to clean
Single-layer chip (10-channel)	T-junction	PDMS	- Easy to fabricate - Monodisperse droplet formation at T-junctions	- Complicated design - Connections between two chips are difficult to make - Droplets clogged in the collection part

Single-layer chip (5-channel)	T-junction	PDMS/ Polycarbonate (Connector)	<ul style="list-style-type: none"> - Easy to fabricate - High droplet monodispersity 	<ul style="list-style-type: none"> - Complicated design; due to the variation in needle shapes at the outlets, PDMS breaking led to leakage - Coalescence at the droplet collection channel
Circular chip (Design 1)	Step emulsification	PDMS	<ul style="list-style-type: none"> - Easy fabrication - Simpler design - monodisperse droplet production 	<ul style="list-style-type: none"> - Droplet aggregation near the outlet led to coalescence
Circular chip (Design 2)	Step emulsification	PDMS	<ul style="list-style-type: none"> - Easy to fabricate - High oil and surfactant volume prevented droplet coalescence 	<ul style="list-style-type: none"> - External oil channel led to obstruction of flow and stagnation of droplets; a very small number of droplets could reach the outlet
Circular chip (Design 3)	Step emulsification	PDMS	<ul style="list-style-type: none"> - Easy fabrication - monodisperse droplets formed 	<ul style="list-style-type: none"> - The large depth of the oil channel led to the stacking of droplets in layers and lowered the flow rates
Circular chip (Design 4)	Step emulsification	PDMS	<ul style="list-style-type: none"> - Easy fabrication - High droplet monodispersity - Very high-throughput possible 	<ul style="list-style-type: none"> (applicable for all circular chip designs) - Milling irregularities could slightly affect channel dimensions - Surface smoothening techniques could affect chip bonding to glass, causing leakage

2.2 High-throughput droplet production and monodispersity

2.2.1 Effect of design parameters on droplet diameter

The finalised circular chip design (hereby termed the DPC) was further investigated to check for tentative causes of polydispersity due to the chip design channel-to-channel variations during micromilling. Out of the 64 parallel droplet generator channels, 6 channels were selected (Channel 3, 12, 24, 34, 46, and 56; channel numbering shown in **Figure 1-5.A**) based on differences in dimensions and position within the design. Droplet generation from each of these channels was carefully monitored at a model hydrogel flow rate (Q_H) and oil flow rate (Q_O) of 25 and 125 $\mu\text{L}/\text{min}$ to allow a sufficient amount of oil-surfactant in the continuous phase to avoid coalescence issues.

Out of the 6 channels, the maximum variation in droplet diameters was observed at Channel 34 (**Figure 1-8.A**). This channel is located very close to the outlet, which may be a reason for the variation, considering the frequency of droplet production is lowest at this channel (**Figure 1-8.B**). This was again an effect of droplet crowding as observed from the droplet generation process videos. The presence of droplets from other channels at the oil channel (on their way out of the chip) in front of Channel 34 prevented the droplet formation at this junction in a regular manner. Considering 190 μm as the average droplet diameter, every channel is supposed to generate ~ 110 droplets/minute. However, other than channel 46, all other channels had below-average droplet production frequency. Even though variations in the wedge height (H_w) showed some effect on variabilities of droplet diameters generated from a particular channel but it was not directly correlated (**Figure 1-8.C.i**). The step height (H_s) of each channel had no visible effect on droplet diameters produced from them (**Figure 1-8C.ii**).

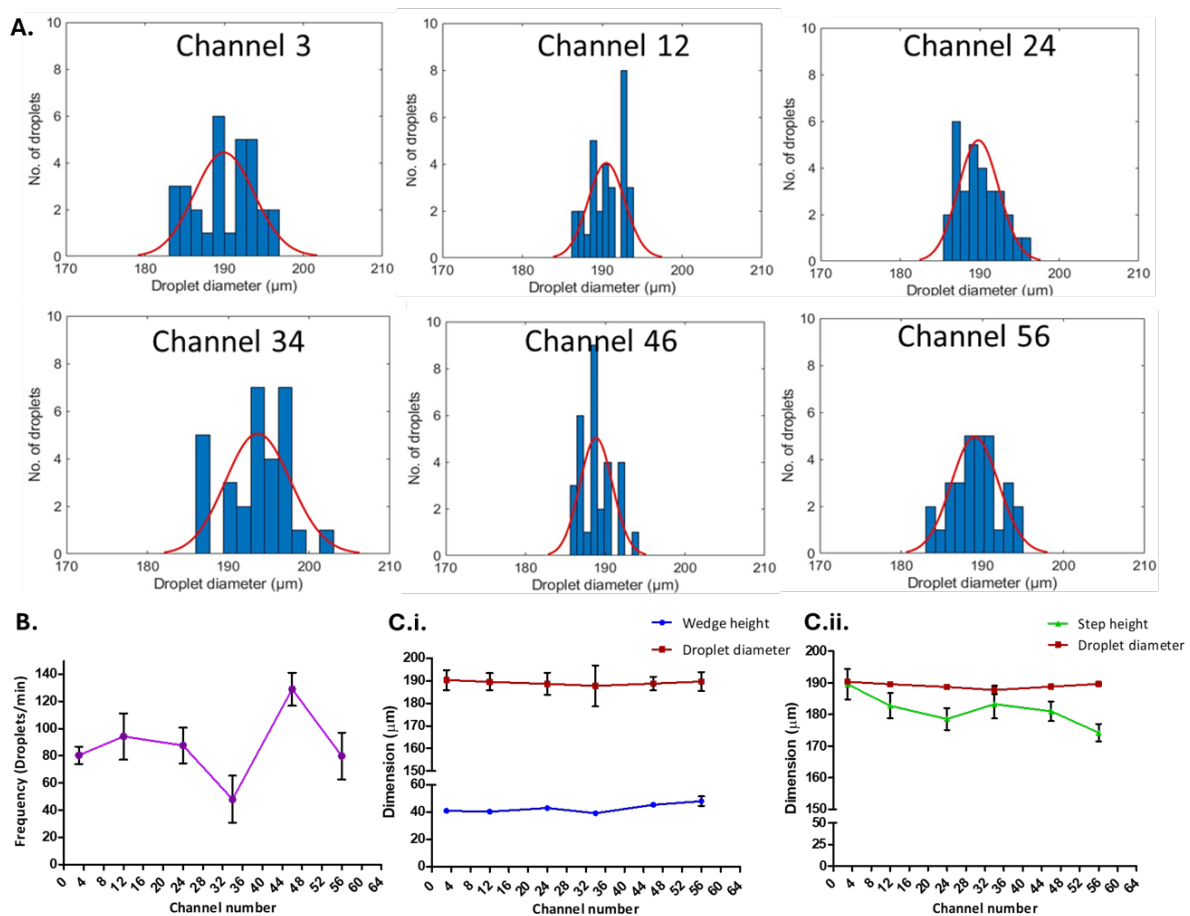


Figure 1-8: Analysis of monodispersity from 6 selected channels. **A.** Histograms depicting variations in droplet diameters from each of the selected channels. **B.** Frequency of droplet generation at $Q_H = 25 \mu\text{L/min}$ and $Q_O = 125 \mu\text{L/min}$ from the 6 channels. **C.** Comparison of the diameters of droplets generated based on i. the wedge height and ii. the step height of selected channels.

Altogether, the position of channels within the chip led to the maximum effect on the polydispersity of droplets generated. The effect of micromilling did affect the droplet generation, but not very significantly. These results led to further investigation into probable factors for polydispersity, such as the continuous phase to hydrogel phase volume fraction.

2.2.2 Effect of volume fraction on droplet monodispersity

For the previous experiments, a large volume fraction of oil-surfactant (5:1 with the hydrogel phase) was used to avoid droplet coalescence at all costs. However, from previous observations, a low volume of oil-surfactant led to droplet coalescence, which plays a limiting role in determining the optimum flow rates for attaining high-throughputs without affecting monodispersity. A total flow rate of 100 $\mu\text{L}/\text{min}$ was maintained, and three volume fractions ($Q_H - Q_O$) of 1:3 (25-75 $\mu\text{L}/\text{min}$), 1:1 (50-50 $\mu\text{L}/\text{min}$), and 3:1 (75-25 $\mu\text{L}/\text{min}$) were tested (**Figure 1-9A.i., ii., iii., and B**). When the fraction of oil was three times higher than the hydrogel, droplets with the highest monodispersity were produced. When the volume fraction of oil and hydrogel is equal, droplet coalescence problems start occurring at the outlet, causing polydispersity in the droplets produced. The droplet coalescence becomes much greater when the oil fraction is further reduced, and even results in the breaking of droplets into smaller droplets, resulting in high degrees of polydispersity.

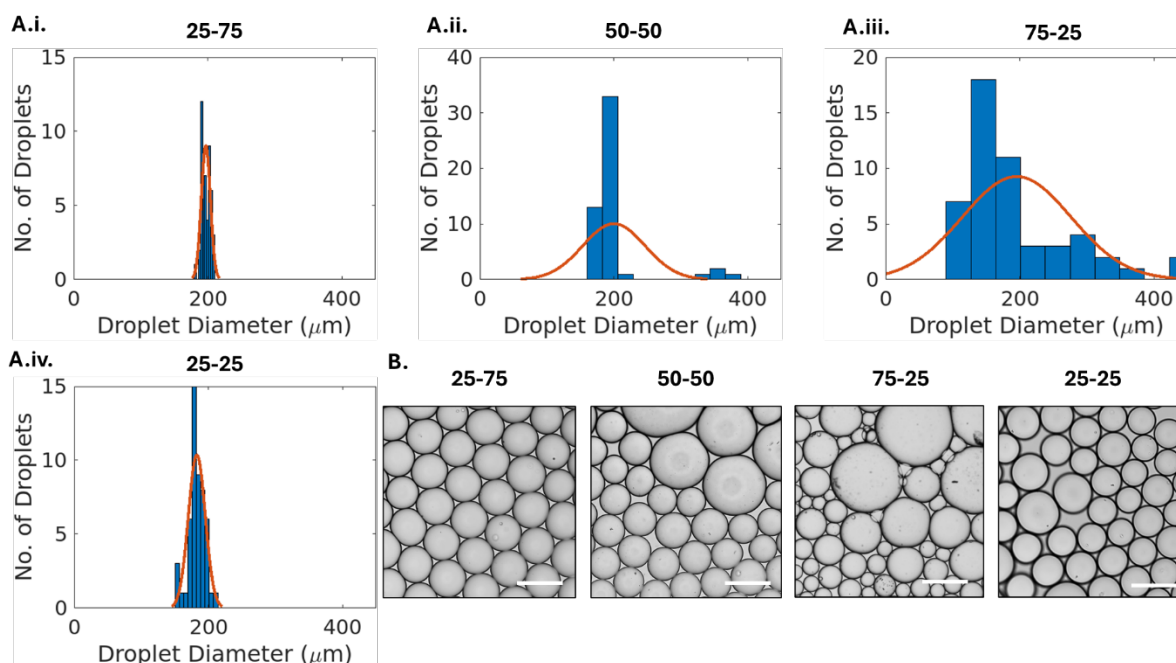


Figure 1-9: Effect of volume fraction on droplet monodispersity. **A.** Histograms showing variations in droplet diameter for the volume fractions $Q_H - Q_O$ as follows: i. 25-75, ii. 50-50, iii. 75-25, and iv. 25-25. **B.** Droplets produced at the respective volume fractions viewed under an inverted microscope (10x objective, Scale bar = 200 μm).

Another case was tested for the 1:1 volume fraction with $Q_H - Q_O$ of 25-25 $\mu\text{L}/\text{min}$ (half of the flow rate previously used; **Figure 1-9A.iv and B**). It resulted in a reduction of droplet coalescence; however, the crowding near the outlet caused production of bigger/smaller droplets, resulting in polydispersity. It was hence inferred that a minimum of two times higher volume fraction of oil to hydrogel was required for monodisperse droplet production while maintaining high flow rates for high-throughput.

2.2.3 Flow rate optimisation for high throughput droplet production

Several flow rates, both for the hydrogel and oil, were attempted to detect the limitations of droplet production from the finalized chip design. Three oil flow rates (Q_O) of 25, 75, and 125 $\mu\text{L}/\text{min}$ were checked, and for each Q_O , 5 hydrogel flow rates (Q_H) of 6, 12.5, 25, 50, and 75 $\mu\text{L}/\text{min}$ were investigated (**Figure 1-10**). For all conditions with Q_O at least twice that of Q_H produced quite monodisperse droplets as expected from previous results. At the lowest Q_H of 6 $\mu\text{L}/\text{min}$, all Q_O showed a tendency to produce slightly smaller diameter droplets from certain channels. This observation was more prominent as the Q_O increased and possibly happened due to faster pinch-off of droplets before the hydrogel could completely form at the wedge in certain high-frequency droplet-generating channels.

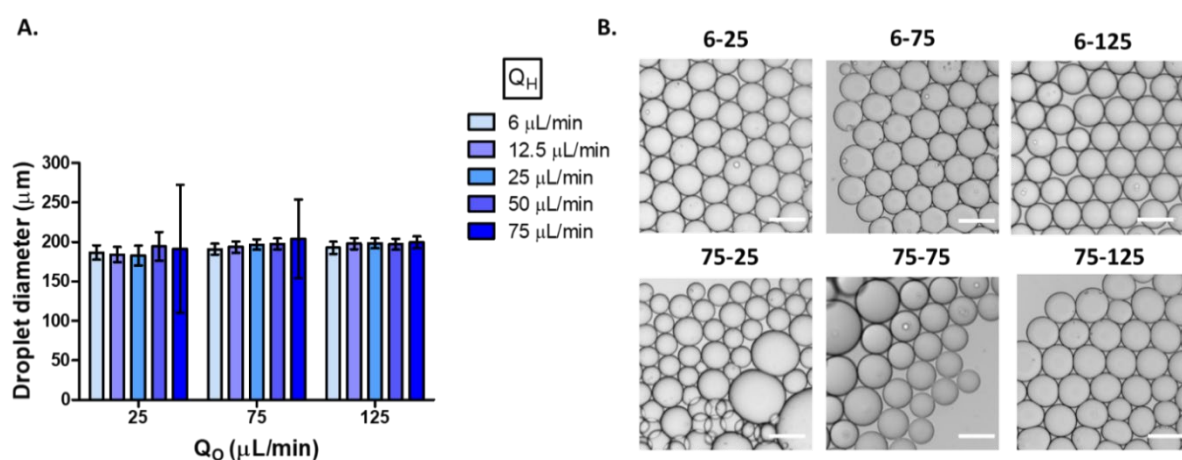


Figure 1-10: Effect of flow rate on droplet monodispersity. **A.** Graph showing average droplet diameter variations for a set of 3 oil flow rates Q_O with 5 corresponding hydrogel flow rates for each. **B.** Droplets produced at minimum and maximum Q_H for each of the 3 Q_O as observed under an inverted phase contrast microscope (10x objective, Scale bar = 200 μm).

The droplet size also did not significantly change as the Q_H , which is commonly observed in other channel designs such as T-junction and flow focusing devices. At Q_H of 6 $\mu\text{L}/\text{min}$, the average droplet diameter was 192.65 (± 7.82), which just increased to 199.52 (± 7.69) at more than ten times higher Q_H of 75 $\mu\text{L}/\text{min}$. Hence, it was confirmed that, other than a low volume fraction of oil to hydrogel, no other effect of flow rates could significantly affect the average diameters of droplets generated.

2.3 Cell Encapsulation in droplets generated in high-throughput

2.3.1 Effect of flow rates on cell-laden droplet diameter and ultra-high droplet production

The droplets were produced under the same conditions of flow rates as in Section 2.2.3, but this time, cells were encapsulated in the droplets. It was checked if the introduction of cells into the system led to differences in droplet diameters (**Figure 1-11A**). No drastic differences were observed when cells were encapsulated in the droplets. A noteworthy observation was made in terms of the process flow based on Q_H . At very low flow rates, the cells had problems with even distribution to all of the 64 channels, as the hydrogel flowing speed was not sufficient to carry the cells to channels further from the initial 16 distribution channels. This resulted in the generation of several empty droplets and, in general, also the number of cells encapsulated

appeared quite low (**Figure 1-11A.ii**). A similar problem with cell distribution was observed at high Q_H values (e.g., 75 $\mu\text{L}/\text{min}$), where the cells showed a tendency to flow towards the wedged channels in closer proximity to the distribution channels, while very few cells were carried to the channels further away. This also resulted in imbalance in cell distribution in droplets and empty droplets. In the optimum Q_H range this problem was observably reduced.

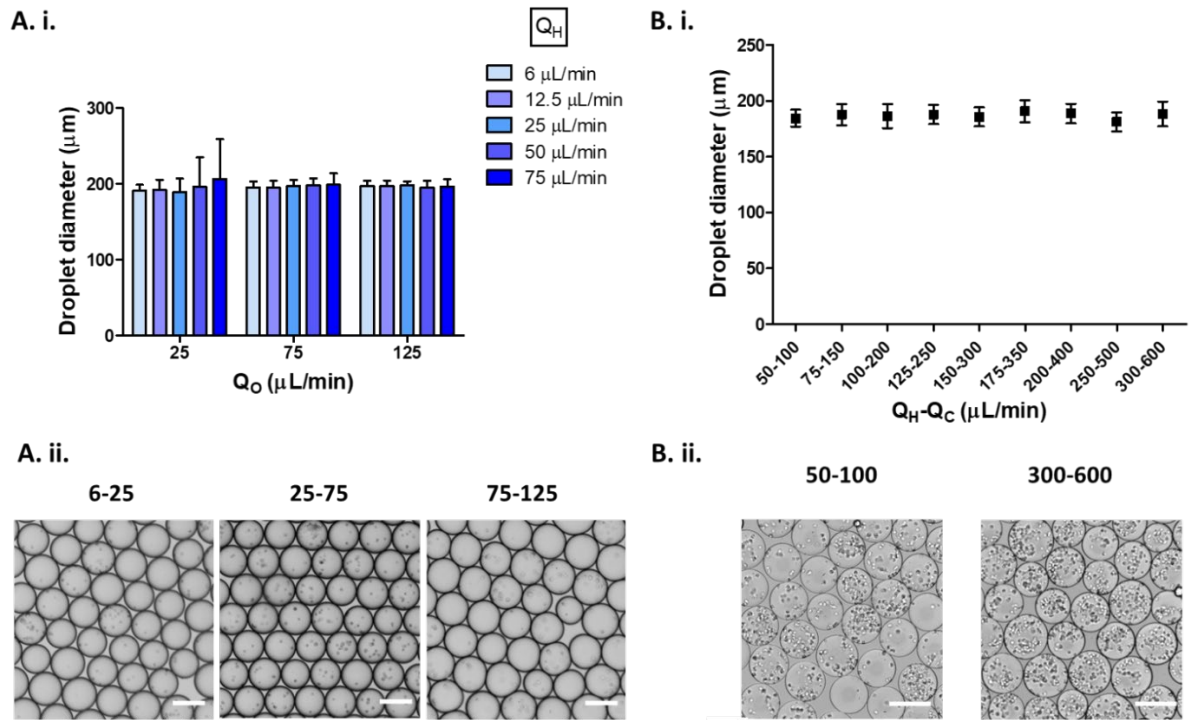


Figure 1-11: Cell encapsulation in high-throughput. **A.i.** Average droplet diameters analysed for the previously tested flow rates for cell encapsulating droplets and **ii.** microscopic images of cell-laden droplets generated at the minimum, optimum, and maximum total flow rate at a cell concentration of 10 million/mL. **B.i.** Droplet diameters compared for very high throughput flow rates and **ii.** microscopic images of the minimum and maximum total flow rates attempted to encapsulate 20 million/mL cells (Scale bar = 200 μm).

To check if cell encapsulated droplets could be produced at higher throughputs, total flow rates ($Q_H + Q_O$) of 150 $\mu\text{L}/\text{min}$ up to 900 $\mu\text{L}/\text{min}$ were tested. It was observed that no significant variations in droplet diameters resulted, even at higher throughputs. According to our tests, the DPC was capable of producing up to about 10^5 cell-laden droplets within a minute, showing ultra-high-throughput capabilities. It is also worth mentioning that the cell loading concentration as high as 20 million cells/mL was used for this experiment without any hindrances with a $Q_O:Q_H$ (2:1). However, our previous theory about high Q_H affecting cell distribution was found to be incorrect. To look into possible factors affecting Q_H leading to cell distribution problems, further investigations were made (results in Section 2.3.2). The only factor that acted as a limitation for high production was when the bonding of the PDMS chip to glass was not strong enough, leading to leakages.

2.3.2 Effect of cell loading concentration and experimental design on cell distribution in cell-laden droplets

Cell loading concentrations of 10 million/mL and 20 million/mL were checked to see how cell distribution changed as a result (**Figure 1-12.A**). High cell loading concentrations were needed for the artificial islets based on the physiological cellular composition of islets in the human body¹². The model flow rates (Q_H - Q_O) of 25-125 $\mu\text{L}/\text{min}$ were used for this experiment to avoid any other factors from affecting the experiment. It was observed that increasing the cell loading concentration helped in avoiding empty droplets, and also the distribution of cells in general improved. Even though in both cases the Poisson distribution was not achievable, at the 20 million/mL cell concentration, the distribution resembled a Poisson distribution with a few outliers. This happens probably due to the design of the chip, as mentioned in the previous section, based on the proximity of the 150 μm distribution channels to each of the 64 wedged droplet generators.

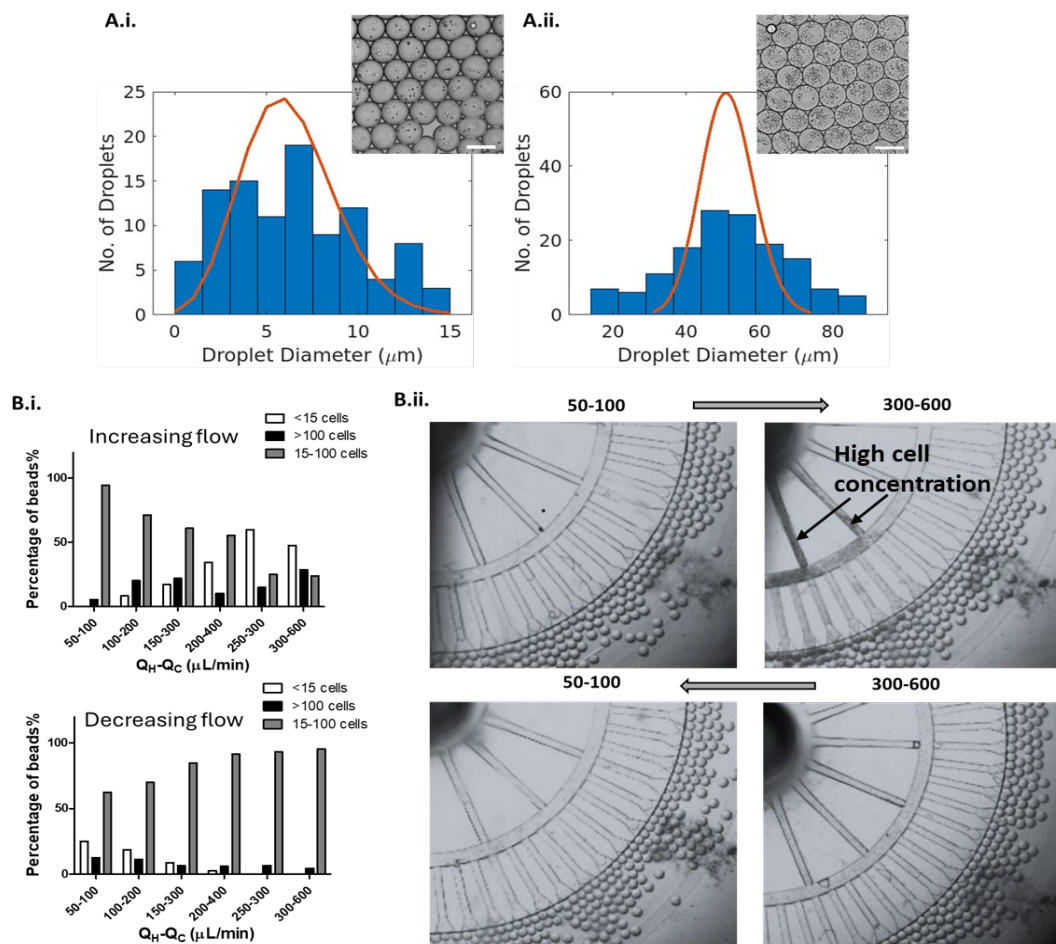


Figure 1-12: Cell distribution in generated droplets. **A.** Histogram showing cell distribution in droplets with a cell loading concentration of **i.** 10 million cells/mL and **ii.** 20 million cells/mL (Microscopic images in insets). Both histograms have been fitted to a Poisson distribution (red). **B.** Sequence of experiments changes **i.** distribution of cells in droplets when performed in increasing and decreasing order of flow rates, and **ii.** cell density translocating from distribution channels to the wedged droplet generator channels.

Based on previous observations, it was checked if the order of experiments performed affected cell distribution in droplets (**Figure 1-12B**). To check this, the ultra-high throughput flow rates were considered as the $Q_O:Q_H$ was constant for all conditions, so the only factor that would affect it would be the changing total flow rates to double. Firstly, for the “increasing flow” case, samples were collected for 30 seconds, and then the flow rates were increased with a 15-second gap between each condition. Samples were collected in the same manner for the “decreasing flow” case, starting from the maximum flow rate condition of 300-600 $\mu\text{L}/\text{min}$. It was surprisingly observed that when flow rates were increased from lower to higher, the cell distribution varied greatly. The cells initially could not get distributed uniformly, and the unevenness just kept magnifying as the flow rate was increased. For the reverse case, the distribution was kind of forced initially due to the very high flow rates, and even though the non-uniform cell distribution effect was still present in the last few samples, but was significantly reduced in magnitude. Also, it was possibly still present due to the constant formation of some droplets with too many cells, resulting in a lesser number of cells available towards the end of the experiment. An important observation made during this experiment was the movement of cells into the distribution channels due to “flow inertia” during the 15-second buffer periods. This could possibly play an additive role in increasing the non-uniformity of cell distribution based on the position of the channels on the chip. Already, channel positions were found to affect droplet production frequency and droplet diameters earlier in Section 2.2.2.

3 Discussion

[High-throughput monodisperse droplet generation reported to date for multi-cell encapsulation]

Among parallel T-junction channels developed for high-throughput microdroplet generation, the work done by Headen *et al.* in 2018 is of great significance¹³. They used a 2-layer PDMS T-junction chip to increase throughput by a huge margin of 600% compared to a single-junction design. The droplet diameters for these droplets ranged from 35-100 μm with a maximum cell loading capacity of 5 million cells/mL to preserve droplet monodispersity. For our T-junction chip, the design complexities resulted in hindrances to a smooth droplet production process. In our step emulsification system, however, the droplet monodispersity was preserved even at a concentration of 20 million cells/mL. A 3D T-junction geometry similar to our initial 10-channel device was demonstrated for high-throughput monodisperse droplet generation by Gelin *et al.*(2020)¹⁴. A double T-junction design with parallel channels was used by Schoeman *et al.* (2014) to achieve droplet production rates greater than 2000 Hz, but was used for single cell encapsulation, unlike our system¹⁵.

[Step emulsification devices for high-throughput droplet generation and cell encapsulation]

High-throughput production of droplets for cell encapsulation applications was generated using a modified step-emulsification microfluidic design. A step T-junction system was used by Ling and colleagues (2022) to obtain high-throughput encapsulation in alginate-gelatin droplets produced at a rate of 1228 droplets/s (or 2400 HepG2 cells/s)¹⁶. Opalski and colleagues developed a grooved step-emulsification channel to add tunability to droplet sizes, which otherwise is not achievable in such step-emulsification¹⁷. An electrowetting-assisted system was developed by He and colleagues to improve monodispersity and throughput¹⁸.

These studies have been claimed to be applicable to cell encapsulation applications. Step emulsification devices have also been used for generating droplets for encapsulating single cells in each droplet¹⁹.

[High-throughput techniques for Single-cell encapsulation]

Single-cell encapsulation has been widely attempted using microfluidic design, especially flow-focusing. Encapsulation efficiency heavily relies on the Poisson distribution and is an important parameter for single-cell encapsulations. Several researchers, such as Chabert and Viovy (2008), Lagus and Edd (2012), and Kemna *et al.* (2012), have reported 70-85% cell encapsulation efficiency using flow-focusing channels²⁰⁻²². Among other designs attempted, a recent research showed crossflow design with sample enrichment, with similar encapsulation efficiency, resulted in high cell viability, while a hydrodynamic micro-vortex was also tested for such applications^{23,24}. Logically, these droplets were of picolitre volume²⁵ compared to our larger, nanolitre-volume droplets, since we needed multiple cells to be encapsulated. Also, in our case, the Poisson distribution influences the distribution of cells per droplet instead of the efficiency.

[Rapid droplet generation using step emulsification and other designs]

Step emulsification-based devices have the capability to maintain droplet diameter irrespective of the flow rate based on device geometry only. A “millipede” device was designed based on step-emulsification by Amstad and colleagues with 550 nozzles for making highly monodisperse PEG droplets (20-160 μm in size) at rates of up to 150 mL/hour²⁶. Similarly, A 364 parallel channel step-emulsification device was developed in glass to produce highly monodisperse droplets with high chemical/thermal stability at rates of 25 mL/hour²⁷. In another study, a 512-channel droplet generator device was used to form fairly monodisperse droplets by droplet splitting at rates of up to 42.7 kHz²⁸. An initial flow focusing generator led to multiple branches of a T-junction type for the droplet splitting. Parallel channels in circular arrangements (as in our design) with >100 droplet generators were used to demonstrate droplet production at rates of up to 1L/hour^{29,30}. None of these devices was used for cell encapsulation purposes at such high rates of production as our system. A bubble-triggered droplet generation technique was demonstrated by Yan *et al.* (2017) with wide channels to prevent cell clogging and rapid production of monodisperse droplets with encapsulated cells, but had a much lower production rate compared to the others described above³¹.

[Possible further investigations]

Further improvements can be made to the chip design to upscale the production further, as seen for some of the research described above. The micromilled devices show non-uniformity of shallow channels, which can be worked upon to obtain originals of the chips through other, more precise fabrication methods or instruments. This can help in lowering the CV even further. The techniques to stabilize these microdroplets for the long term by crosslinking have been discussed in the next chapter.

Chapter 2 : Optimization of Crosslinking Conditions and Development of an On-the-fly Crosslinking System

Droplets formed by water-in-oil emulsification using hydrogels need to be crosslinked in order to convert them into a non-soluble gel phase to remain stable in the cell culture media. Various methods of crosslinking are present which require their own optimizations and configurations. Once crosslinked the microbeads can be used for 3D cell culture and tissue engineering applications.

Traditional crosslinking techniques

Crosslinking also known as curing or polymerization is a process to modify prepolymer solutions (sol) into durable porous materials (gels) with enhanced physico-chemical properties. The traditional crosslinking methods use physical (intermolecular forces are manipulated to form stable (mostly reversible) structures) or chemical (using external chemical agents to trigger an irreversible reaction) techniques. The table below (**Table 2-1**) gives a brief summary of the different crosslinking methods traditionally used^{32,33}.

Table 2-1: Traditionally used techniques for hydrogel crosslinking.

Physical crosslinking				
Methods	Brief description	Sub-categories	Examples	References
Intermolecular interactions	Coordination bonds or complexes are formed between certain residues due to the presence of ions or opposing charges	Ionic	Alginate crosslinking using divalent ions like Ca^{2+}	[1], [2] ^{34,35}
		Electrostatic	Chitosan forming polyelectrolytic complexes with anionic polyelectrolytes like pectin, cellulose, polylactic acid	
Physical factor-induced	The external factors induce the rearrangement of the molecules forcing the sol-gel phase transition	Temperature-induced	Derivatives of poly(<i>N</i> -isopropylacrylamide) form thermo-responsive hydrogels	[3],[4] ^{36,37}
		Ultrasonic-mediated	Silk fibroin forms β -sheet structures on sonication	
Hydrogen bonding	Non-covalent interaction forming a H-bond to stabilize or form structures	Secondary-structure stability	Agarose gels formed	[5],[6] ^{38,39}
		Structure formation	H-bond formed between O-group of PEG and COOH-group of polyacrylic acid	

Crystallization	Crosslinking happens by the physical control of conditions to form a crystalline hydrogel structure	Homopolymer crystallites act as crosslinkers	PVA solution with sodium alginate after repeated freeze-thawing cycles forms a highly tensile hydrogel	[7],[8] ^{40,41}
		Stereo-complex formation between isomers	The D and L enantiomers of Polylactic acid can have a racemic interaction to form a composite hydrogel	
Metal coordination	Metal ions interact with the polymeric units which act as ligands		Poly(acrylamide- <i>co</i> -acrylic acid) copolymer crosslinking in FeCl ₃ solution	[9] ⁴²
Self-assembly	In amphiphilic molecules, the hydrophilic parts in an aqueous medium trigger the hydrophobic parts to arrange automatically forming micelles		Double hydrophilic block co-polymers with polyethylene(oxide) or acylamide-based diblock copolymers	[10], [11] ^{43,44}
Protein interactions	Selectively cross-linkable materials are synthesized by utilizing peptide interaction in the polymer	Antigen-antibody interaction	An anti-concanavalin A antibody was grafted to poly <i>N</i> -isopropylacrylamide	[12],[13] ^{45,46}
		Genetically engineered	A stimuli-responsive phenothiazine hydrogel was produced with genetically modified calmodulin	
Chemical crosslinking				
Methods	Brief description	Sub-categories	Examples	References
Photo-polymerization	Light-induced polymerization of the hydrogel monomers in the presence of a photoinitiator	Free-radical	Methacrylated gelatin or dextran with photoinitiators like LAP and Irgacure	[14], [15],[16] ^{47–49}
		Thiol-ene	Norbornene-modified hyaluronic acid (HA) with dithiothreitol (DTT) as crosslinker and Irgacure 2959 as photoinitiator	
		Redox	Tyramine conjugated HA photo-cross-linked in the presence of rose bengal and eosin-Y photoinitiators	

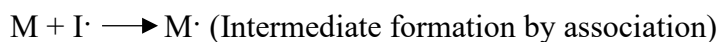
Enzyme-catalyzed reactions	Enzymes are used to kinetically control and catalyze the covalent bond formation reaction		Tyramine-conjugated collagen was crosslinked using horseradish peroxidase enzyme in the presence of H ₂ O ₂	[17] ⁵⁰
“Click Chemistry” - based	This technique allows reactions to be performed in an atom-efficient, selective manner to result in high product yields	Diels-Alder reaction	Reaction of PEG-maleimide with methylfuran	[18],[19],[20],[21] ⁵¹⁻⁵⁴
		Michael’s addition reaction	DTT and glycidyl methacrylate-conjugated dextran were reacted in PBS solvent	
		Schiff base formation	Reaction between the amino and dialdehyde groups from acrylamide-modified chitin and oxidized alginate, respectively	
		Oxime linkages	Dimethylacrylamide and diacetone acrylamide copolymers crosslinked by difunctional alkoxyamines	
Reversible covalent linkages	Based on pH compared to pK _a results in esterification (especially of boronic acid derivatives) or alternatively in its free form		Boronic acid was conjugated to alginate, resulting in a reversible complex formation of boronate-cis-diol	[22] ⁵⁵
High-energy radiations	Gamma rays or electron beam can be used for crosslinking		Tara gum/acrylic acid - based polymers were crosslinked in the presence of methylenebisacrylamide using gamma rays	[23] ⁵⁶

Photo-crosslinking: Free radical polymerization

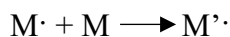
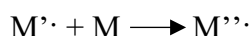
Photo-crosslinking traditionally is performed based on free radical polymerization. Compounds with functional groups such as methacrylate, acrylate, acrylamide, and similar are suitable for such photo-crosslinking reactions⁵⁷. The reaction follows a three-step product formation process including:

Step 1: Initiation - Free radicals are formed by the photolysis of the photoinitiator molecule (I). The generated radicals covalently react with certain functional groups in the polymer

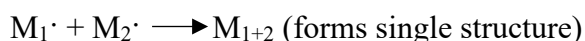
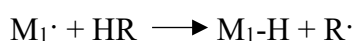
structure (M) to form reaction intermediates. The propagation mostly takes place through π -bonds.



Step 2: Propagation – The reaction intermediates that are formed attach to other reactive groups available and transfer from molecule to molecule forming a chain that keeps growing as the reaction proceeds.



Step 3: Termination – The final step of the reaction involves the formation of the polymer with crosslinked monomer linkages. Termination can occur either by joining two radical bearing ends (radical coupling), by neutralizing the terminal groups with saturation in one and unsaturation in the other (disproportionation), or by transfer of the radical away from the polymer chain (chain transfer).



The photo-crosslinking process is considered as a second-order kinetic reaction. The rate of polymerization (R_{pol}) can be obtained by the formula:

$$R_{pol} = k_{propagation} \sqrt{\left(\frac{\phi \epsilon I_i}{k_{termination}}\right) \cdot [I] \cdot [M]}$$

Where,

$k_{propagation}$ is the rate constant for the propagation step,

$k_{termination}$ is the rate constant for the termination step,

ϕ is the quantum yield,

ϵ is the photoinitiator's molar extinction coefficient,

I_i is the incident light intensity, and

$[M]$ and $[I]$ are the concentrations of unreacted functional groups and the photoinitiator, respectively.

Advantages and disadvantages of the process

Photo-crosslinking as a technique has been widely used for biomedical applications due to its several advantages. However, like all processes, it has certain limitations as well. **Table 2-2** gives a precise list of the advantages and disadvantages of the process⁵⁸⁻⁶⁰:

Table 2-2: The advantages and limitations of the photo-crosslinking process.

Advantages	Disadvantages
Rapid process, can be completed in a matter of seconds thereby reducing experimental time	Most natural polymers lack the photocrosslinkable groups in their inherent structure
Can be performed using even visible light without significant photodamage to cells	Conjugation of photocrosslinkable groups possibly induces cytotoxic features
Can be used for bioprinting and injectable applications and can be performed <i>in vivo</i>	Incomplete process can result in residual free radicals inducing oxidative stress in cells
Easy control of process without need for tedious mixing steps or long exposure times; highly stable structures formed	Not many water-soluble, biocompatible photoinitiators are present thereby limiting the options
Can be selectively performed on a single structure by directing light to specific spots; can be combined with other crosslinking methods	Visible light-crosslinked hydrogels are highly sensitive to external lighting conditions and need to be protected from light until the crosslinking is performed

GelMA crosslinking mechanism

The crosslinking of GelMA follows the same mechanism of photo-crosslinking as described above. The Li-chelated photoinitiator, lithium phenyl-2,4,6-trimethylbenzoylphosphine (LAP), forms two free radical molecules when irradiated with UV/blue light (350 – 410 nm, with peak absorbance values near 375-380 nm)⁶¹. These free radicals interact with the C = C bond on the methacrylate group and continue the chain propagation (**Figure 2-1**).

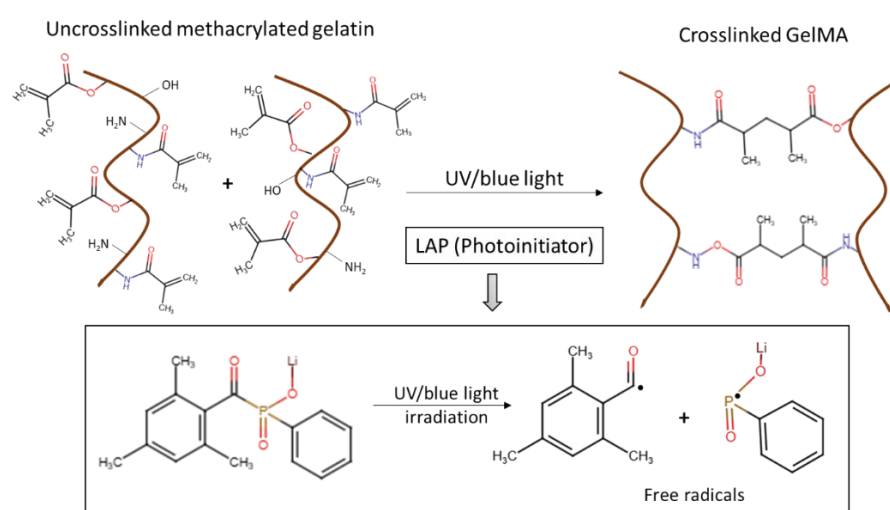


Figure 2-1: Photo-crosslinking of GelMA in presence of photoinitiator LAP (Photocatalysis of LAP shown in the inset).

As seen for most of the free radical photo-polymerization reactions, GelMA crosslinking also heavily depends on the crosslinking light intensity, the photoinitiator's concentration and ability to form free radicals, and the presence of reactive groups on the polymer. The latter determines the stiffness and degradability of the crosslinked hydrogel; the higher the methacrylation of gelatin, the stiffer the crosslinked hydrogel, as more linkages are possible due to the presence of more double-bonded structures⁶². Crosslinking efficiency of GelMA can be analysed based on rheological analysis, NMR, or colorimetric analyses like TNBS assay⁶³.

Cell encapsulation in crosslinked droplets: advantages

Droplets of spherical geometry provide a huge space within their structure while occupying a minimum surface on the outside⁶⁴. The absence of corners in a sphere helps avoid bias in the assembling or localization of cells⁶⁵. In the case of crosslinked hydrogel droplets, the system becomes even more efficient due to the fibrous interconnections formed within, providing enough surface for the cells to attach and grow on⁶⁶. Unlike 2D cell culture systems, the cells can be in 3D space thanks to the porous nature of the crosslinked hydrogels. The pores allow the exchange of gases, nutrients, and wastes into and out of the droplets⁶⁷. Additionally, due to the crosslinking, the structure is maintained for a significant period to observe cell behaviour over time⁶⁸.

This chapter deals with optimizing the conditions for crosslinking of the generated droplets. Once the droplet production chip (DPC) design was confirmed, an on-chip crosslinking setup was integrated with the system so that produced droplets could be crosslinked “on-the-fly” (OTF). Another set of optimizations was performed at this point. The crosslinking light wavelength (385 nm) and irradiance (884 mW/cm²) were thoroughly optimized based on the best crosslinking and highest cell viability. Also, the crosslinking chip (C-chip) channel length and flow rate were optimized to obtain the perfect exposure time (15 s). Additionally, a black acrylic box fitted with a heat-dissipating fan was designed to facilitate better process control for the crosslinking setup. The finally developed system was used to produce crosslinked, cell-encapsulated droplets at a rate of around 8000 droplets /min with an average of 50 cells per droplet for a cell concentration of 20 million cells/mL of hydrogel.

1 Materials and Methods

1.1 Sample preparation

1.1.1 Cell culture

Mouse fibroblasts, L929, were used for all optimization studies. The cells were cultured in T75 flasks with complete media composed of: DMEM GlutaMax media with 1% Anti-anti (antibiotic-antimycotic) and 10% FBS (Fetal bovine serum, heat-inactivated). For experiments and sub-culture, the spent medium was removed, and cells were washed with 1X PBS. After removing PBS, 2 mL of Trypsin was added and incubated in the incubator at 37°C, 5% CO₂ for 3 minutes. After removing from the incubator, 5 mL of whole media was added to the detached cells and mixed gently several times before collecting the cell stock in a 15 mL Falcon tube. The cell stock was centrifuged at 1200 rpm for 5 minutes. All cell culture reagents were obtained from Gibco (ThermoFisher GmbH, Germany).

For sub-culture, after centrifugation, the media above the cell pellet was carefully removed and replaced with fresh whole media. The media and the cells were gently mixed together with a pipette to thoroughly resuspend the cells in the media and break cell clusters. Depending on the cell density of the detached cells, the resuspended cells in media were placed in 10 mL of fresh whole media in a T75 flask and placed in the incubator. Sub-culture of cells was done every 3-4 days.

For the experiments, the cells after centrifugation were resuspended in 1 mL of whole medium. From this stock, 10 μ L of the cell stock was mixed with 10 μ L of Trypan blue stain, and 10 μ L of this mix was added to a Countess cell counter slide. The slide was inserted into a Countess cell counter (NanoEntek, Germany) to measure the cell concentration. Accordingly, cell stock was taken for 10 million cells or 20 million cells per mL in the final hydrogel solution.

1.1.2 Hydrogel preparation

The freeze-dried GelMA was dissolved in PBS at the desired concentration by stirring for 1 h at 50°C. A LAP photoinitiator stock was prepared at 4% w/v in 70% ethanol. This LAP solution was added at 0.4 % concentration by volume to the hydrogel solution and filtered using a 0.22 μ m filter (Millipore, Sigma GmbH, Germany). For cell encapsulation experiments, the cell stock and the hydrogel pre-polymer solution were mixed in a 1:4 v/v ratio.

1.2 Droplet crosslinking methods

Droplets were generated according to the method described in Chapter 1 (Materials and Methods; Section 1.4). For these droplets, two methods of crosslinking were attempted: stationary crosslinking and “on-the-fly” crosslinking.

For stationary crosslinking, the generated droplets were collected from the outlet of the chip into a 3 cm diameter petri dish and placed on top of an LED UV lamp (385 nm or 405 nm) as shown in **Figure 2-2.A.i**. Alternatively, the droplets were collected into a 1.5 mL Eppendorf and illuminated with UV light using a waveguide-mediated point source (Omnicure lamp; **Figure 2-2.A.ii**).

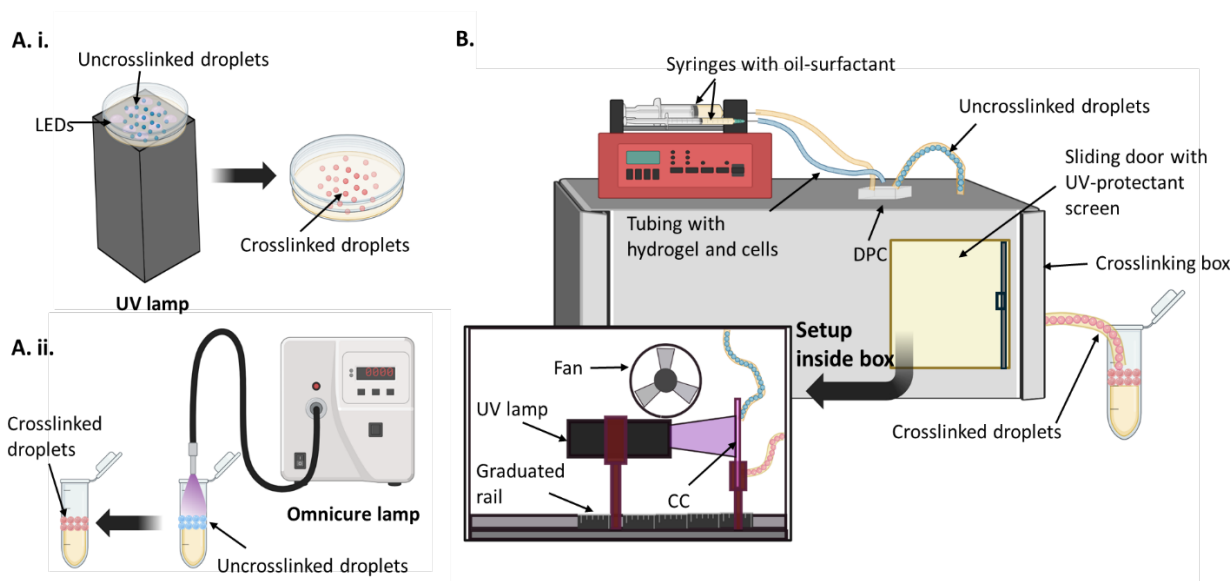


Figure 2-2: The schematics of the system design for **A.** stationary crosslinking using **i.** LED lamp and **ii.** Omnicure lamp; **B.** On-the-fly crosslinking with the arrangement of the lamp and chip inside the box (inset).

For the “on-the-fly” crosslinking, the outlet channel of the DPC was connected to the inlet of a crosslinking chip (CC) using PE tubing. The CC was placed on a holder in an opaque, black PMMA box in front of the UV LED lamp mounted on another holder (**Figure 2-2.B**). The box was fitted with a fan to dissipate heat during the process. As the droplets passed through the CC, they got crosslinked. The final droplets were collected from the CC outlet using a tubing. The design of the CC and the box has been described in the results section. The CC was fabricated according to the methods of DPC fabrication as described in Chapter 1 (Materials and methods, Section 1.1.2.).

1.3 Washing droplets

To transfer the crosslinked droplets into an aqueous phase, the droplets were washed using a 3-step process to remove oil and surfactant. Firstly, Novec 7500 with 20% Perfluorooctanol (PFO) was used to remove the surfactant rapidly, leading to the formation of a single aggregated blob of droplets. The PFO solution was taken out, and pure Novec 7500 was used twice to wash the aggregate. Finally, PBS was added to the aggregate and centrifuged for 5 min at 1200 rpm. Post-centrifugation, the PBS and oil from above and below the droplet layer, respectively, were removed with a pipette. This PBS washing step was repeated three times. The finally obtained droplets were either transferred to cell culture media or to fresh PBS and placed in the incubator at 37°C.

1.4 Lamp power and temperature measurement

Four lamps were used for the crosslinking optimization studies: a custom-made violet-blue light (405 nm) LED lamp (referred to as CML) assembled in the laboratory, a commercially available lamp used for cosmetic application like nail curing (referred to as NCL, SUNone, Leobert, Poland), and two commercial high-power lamps of 405 nm and 385 nm wavelength specific light (referred to as CL₄₀₅ and CL₃₈₅ respectively; from LucBei, China). The CML

consisted of a square array of $5 \times 5 = 25$ LEDs at 5 mm spacing between the LEDs and a fan was fitted to the lamp for preventing overheating of the LEDs. Both the CLs had a similar make with a square array of $4 \times 4 = 16$ LEDs capable of producing high-power light. The PM 100D (Thorlabs, Inc., United States) device was used to measure the irradiance power of the different lamps. The optical power meter sensor was placed at the required distance in line with the LED array of the lamps using the CC holder which was mounted on a graduated rail. The irradiance of the lamps was measured using the following formula:

$$\text{Irradiance} \left(\frac{\text{mW}}{\text{cm}^2} \right) = \frac{\text{Power measured by sensor (mW)}}{\text{Area of the sensor aperture (cm}^2\text{)}}$$

For the temperature measurements, a miniaturized PT100 sensing element (1 mm) was fitted with a thermocouple relay device with a display to act as a thermosensor. The sensing element was fitted in between the PDMS chip and the glass covering the channels. Alternatively, a thermosensor with a pointed PT100 probe was fixed at the outlet of the CC to detect the temperature of the oil after the UV exposure.

1.5 Evaluation of crosslinking

Droplets were analysed using the inverted microscope (Nikon Eclipse AS1). After image acquisition, the distance measurement tool on NIS-Elements software was used to analyse droplet features. ImageJ (Fiji) was also used to enhance the contrast of images to clearly distinguish between the differently cross-linked regions of the droplets.

1.6 Rheological characterization

The rheological analysis of the hydrogel in oil or hydrogel was performed using an optical rheometer of the model Kinexus PRO (Malvern, US). Around 700 μL of the hydrogel/hydrogel in the oil-surfactant sample was tested for a temperature range of 20-60 $^{\circ}\text{C}$.

1.7 LIVE/DEAD cell staining

Cell viability analysis was performed on encapsulated cells using a LIVE/DEAD kit (Invitrogen, Waltham, MA, USA). Beads with cells were collected in Eppendorf tubes and centrifuged at 1200 rpm for 5 minutes. Carefully, the media from above the beads was removed, and PBS with the dye concentration of 3:10 (3 parts of the dye in 10 parts of PBS) was added to the beads. The dye (containing BOBO-1 Iodide and Calcein AM dyes for dead and live cells, respectively) was allowed to enter the cells by incubating for 15 min at 37 $^{\circ}\text{C}$. A part of the bead solution was placed on a microscope glass slide, and images were taken at 10 $\times$ magnification using a green fluorescence channel (480 nm excitation) for live cells and a red fluorescence channel (560 nm excitation) for dead cells using Nikon Eclipse TSR2 or a confocal microscope Eclipse Ti2 (Nikon, Tokyo, Japan). Images were analysed and visualized using NIS-Elements software (Nikon).

2 Results and Inferences

2.1 System design for on-the-fly crosslinking

2.1.1 The crosslinking chip design

The on-the-fly crosslinking system was developed during the project to enable uniform crosslinking of droplets and better overall control over the experimental methods for enhanced reproducibility. The droplet production system was left unchanged. To the outlet tubing of the DPC, a crosslinking chip (CC) was connected. The chip had a serpentine channel of a total length of 67.5 cm with 13 turns. This structure enabled providing the time required for the droplets to get crosslinked completely while saving space. The channel had both a width and depth of 300 μm to sufficiently accommodate the approximately 200 μm droplets (**Figure 2-3.B**).

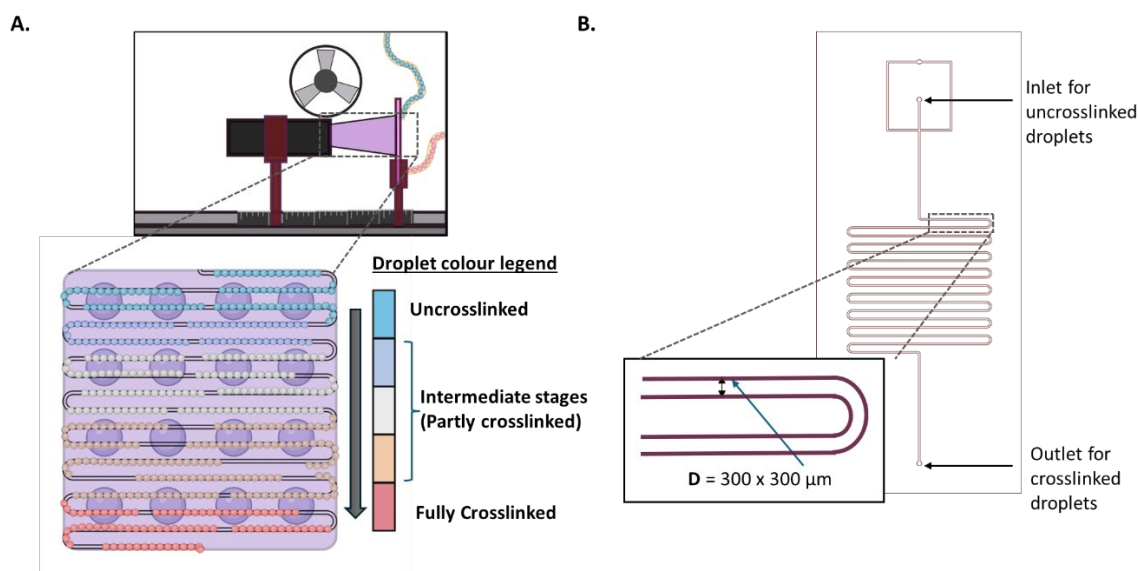


Figure 2-3: On-the-fly crosslinking system. **A.** Free radical polymerization of the droplets as they flow through the CC while being exposed to UV light from the LED lamp. **B.** Design of the CC with channel dimensions (inset).

As the uncrosslinked droplets pass through the illumination zone within the serpentine channels of the CC, the free radical polymerization process takes place. Like all polymerization reactions, chain growth takes place from all available monomer units (described as the intermediate phase in **Figure 2-3.A.**). At the final stage, almost all of the monomer units have undergone polymerization (in this case, crosslinking) to make a stable structure. For obtaining well crosslinked droplets, the time of exposure and light irradiance, and wavelength play a very important role. In the next section, these optimization studies will be discussed in detail.

2.1.2 Initial analysis of crosslinking using the OTF system

Initially, 405 nm was considered to be the ideal wavelength for crosslinking the droplets based on previous research. An LED array of 5 x 5 using 405 nm lights was assembled in the laboratory (CML) with a current input ranging from 0.5A to 3A (50-255 mW/cm^2 irradiance;

Figure 2-4.A). The lamp was fitted with a fan for heat dissipation. For comparison, a commercially available NCL was used with dispersed LEDs of 365 nm and 405 nm auto-switching lights with power modes of 24W and 48W (**Figure 2-4.A**). The OTF system was checked using these lamps initially. These experiments were performed without the crosslinking box by placing the CC on the CML or inside the NCL. A flow rate of Q_{H-Q_0} of 25-75 $\mu\text{L}/\text{min}$ was used for this experiment to produce the droplets in the DPC and subsequently, sufficiently crosslink them on the CC.

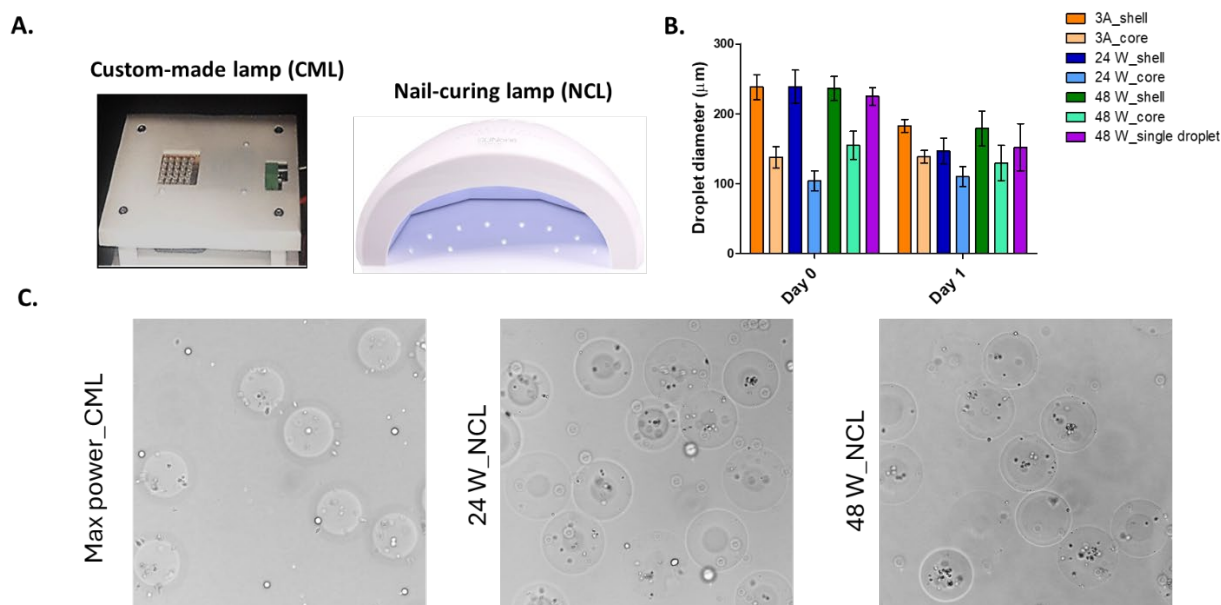


Figure 2-4: Initial crosslinking optimizations. **A.** The lamps used for crosslinking, **B.** Core-shell variations on Day 0 vs Day 1 after crosslinking and **C.** corresponding images for the same, visualized at 10x magnification using a phase-contrast microscope.

It was observed that the NCL at higher power (48W) could produce droplets without a core-shell appearance (**Figure 2-4.B** and **C**). This “shell” -like structure was found to reduce significantly over time, indicating a possibility of incomplete crosslinking in all cases. In terms of stability, the droplets survived a maximum of 72 hours in culture (at 37°C in the incubator). Very tiny or no droplets were visible for any of the conditions after the 3 days, irrespective of the lamp being used. The NCL was found to be less efficient than expected, possibly due to the dispersed LEDs, which prevented the concentration of the light at the serpentine channels, unlike the CML. This can be a reason why the CML performs better even at a lower power. At this stage, it was found that the system was inconsistent, and placing the CC on the lamp directly resulted in overheating. Additionally, the external lighting conditions affected the irradiance available to the CC.

2.1.3 Improvements to the OTF system

To do away with the above-mentioned issues, the “crosslinking box” was designed to enable better control of temperature and distance from the lamp. However, testing with the NCL was discontinued as it was not possible to accommodate the lamp in the crosslinking box. Instead, the commercially obtained CL₃₈₅ and CL₄₀₅ were introduced, which provided a higher range of irradiance compared to the CML. To avoid possible differences in results due to the effect of light wavelength, only the CL₄₀₅ was used for these experiments. The irradiances were

compared for the two lamps, and the effect of the distance of the CC from the lamp for each was analysed. Also, the Q_H - Q_O of 25-125 $\mu\text{L}/\text{min}$ was used based on bulk hydrogel tests (bulk GelMA crosslinked within 15 seconds) and on-chip exposure time calculations while maintaining a sufficiently high volume fraction of oil to hydrogel. Additionally, it was checked how the box prevented interference from external light in the experiment.

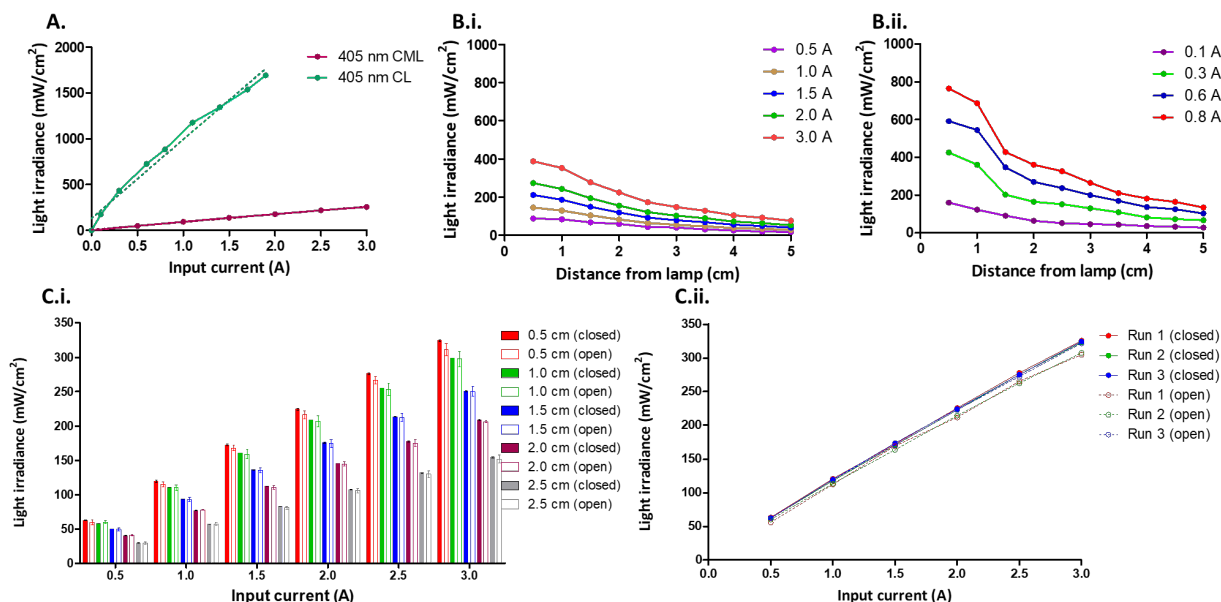


Figure 2-5: Light irradiance optimization based on process control. **A.** Light irradiances measured at minimum distance (0.5 cm) from the lamp in the crosslinking box for the 405 nm CL and CML. **B.** Effect of distance of the lamp from the chip on light irradiance available for crosslinking using **i.** CML and **ii.** CL₄₀₅. **C.** Interference from external light prevented by the box (closed) compared to the open system **i.** for different distances and **ii.** 3 consecutive experimental runs at 0.5 cm distance.

The irradiances for the CL were much higher (175-1692 mW/cm^2) compared to the CML, which could reach a maximum of 255 mW/cm^2 . However, the linearity of the CML was better than the CL (indicated by the dotted lines in **Figure 2-5.A**), indicating a more stable light source based on input current. As for the effect of the distance at which the chip was placed from the lamp, for both the lamps, the change in irradiance became more prominent as the distance was increased as for higher irradiances as expected (**Figure 2-5.B**). At 0.8 A input current, the change is so drastic that at a 5 cm distance, the irradiance falls from 765 mW/cm^2 to 135 mW/cm^2 , reaching the range of the CML. It was hence confirmed that to have maximum irradiance available for crosslinking to the CC, the CC was to be placed at 0.5 cm from the lamp for all future experiments.

The crosslinking box helped in maintaining the illumination reaching the CC. This was proved by checking the irradiance on the CC when the box's top lid was closed vs when it was left open (**Figure 2-5.C.i**). As the distance of the lamp from the CC was increased, the effect of external light became more prominent. For different illumination conditions outside during consecutive experimental runs at the same distance, also variations in irradiance reaching the CC varied, proving the utility of the box against external lighting conditions (**Figure 2-5.C.ii**).

2.2 Optimization of crosslinking light irradiance and wavelength

2.2.1 Higher irradiance can improve crosslinking

Experiments were performed with the complete OTF using the CML and CL₄₀₅. The CC was placed at a fixed distance of 0.5 cm from the lamps. On observing the droplets under the microscope immediately after washing, a similar core-shell appearance of droplets were observed as seen earlier (**Figure 2-4**), where the shell was observed to be partially crosslinked. However, a clear trend was observed in the shell size with respect to irradiance (**Figure 2-6.B**). For the CML, as the irradiance increased, the shell size proportionally decreased almost linearly. For the CL₄₀₅ a similar trend was observed until around 1200 mW/cm², but started increasing for higher irradiances. Altogether, the lowest average shell sizes were observed to be around 29 μm for the 1175 mW/cm².

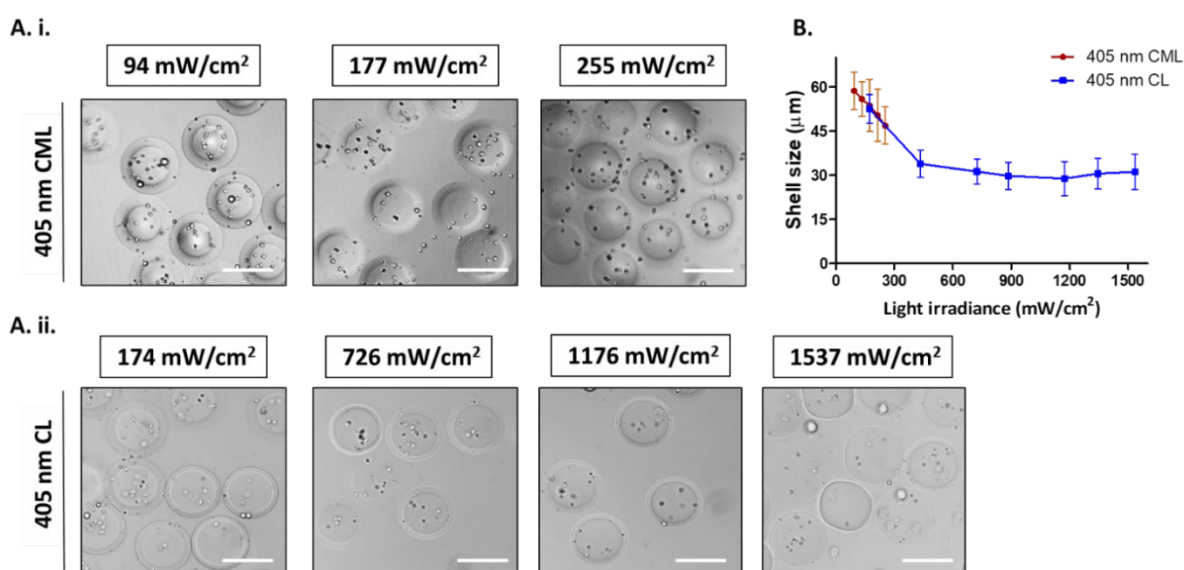


Figure 2-6: Light irradiance optimizations. **A.** More prominent partially crosslinked “shells” observed for droplets crosslinked using **i.** the CML compared to the ones crosslinked with **ii.** the CL (Scale bar = 200 μm). **B.** Size of the shells for each fixed light irradiance available with both the CML and CL.

As seen in the previous case, the droplet shells kept depleting with each day in culture, also for this experiment. The droplet cores remained stable until day 7 of culture, and cells remained encapsulated in them. Interestingly, for the droplets crosslinked at above 1000 mW/cm², even the cores were found to be pretty unstable. By the end of day 3 of culture, the droplets crosslinked at around 1345 mW/cm² and 1535 mW/cm² were completely dissolved, and cells were found floating around in the cell culture media. For the 1175 mW/cm² condition, the droplet sizes were significantly reduced, and some cells were released as well.

2.2.2 Effect of wavelength on crosslinking

To see if crosslinking could be improved by using near UV wavelengths rather than using visible light (405 nm lies in the visible range of the spectrum), a CL of 385 nm wavelength light was used for experiments, and droplets were compared. At around 885 mW/cm² irradiance, a very negligible shell was observed with droplets appearing “almost” completely

crosslinked for the CL₃₈₅ (**Figure 2-7.A**). In general, the shell sizes were almost always smaller for CL₃₈₅ than those of CL₄₀₅ (except for the maximum irradiance, 1537 mW/cm²).

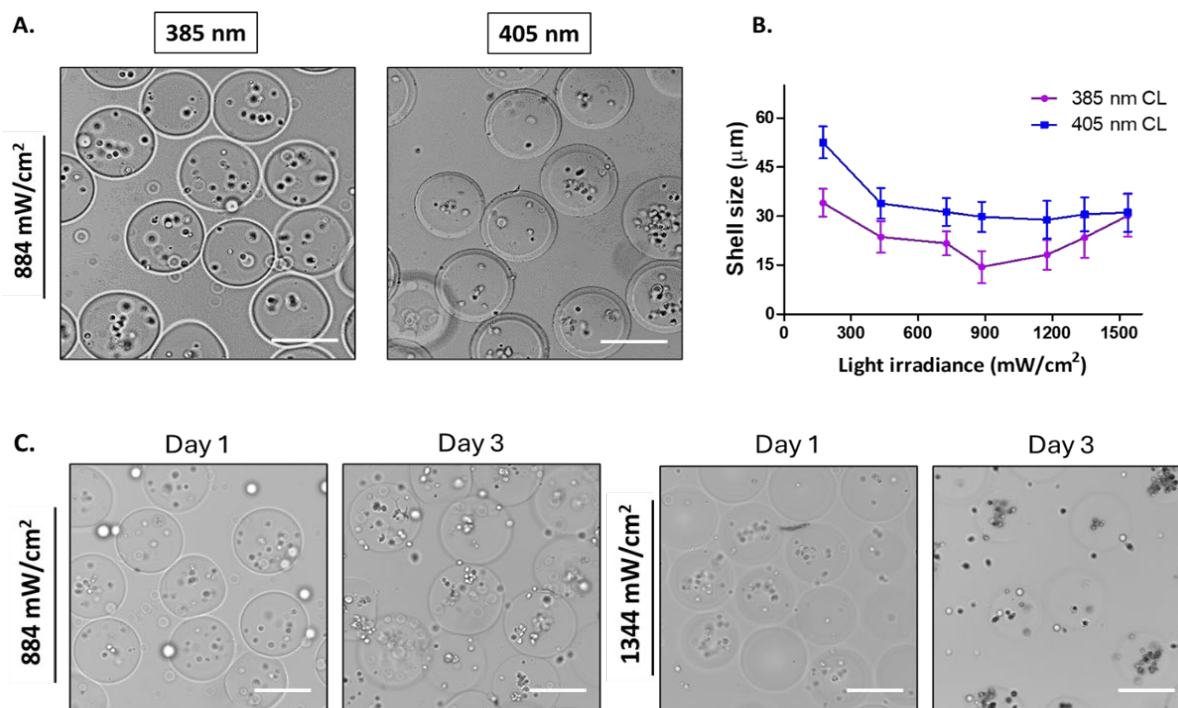


Figure 2-7: Light wavelength optimizations. **A.** Droplets crosslinked at 884 mW/cm² using CL₄₀₅ show bigger partially crosslinked “shells” compared to CL₃₈₅ at the same irradiance (Scale bar = 200 μm). **B.** Shell sizes were observed for the same irradiance intervals for both the CLs. **C.** Droplets dissolving at Day 3 of culture for 1344 mW/cm² crosslinked droplets vs stable droplets crosslinked at 884 mW/cm².

However, a similar trend was observed of increasing shell sizes for irradiances above 1000 mW/cm² and similar instability of droplets (**Figure 2-7.B**). For the CL₃₈₅ the increase was more observable than for CL₄₀₅. Also, for the highest two irradiances, droplets started dissolving after day 3, while the 1175 mW/cm² crosslinked droplets reduced in size. As for the most well-crosslinked droplets, the droplet sizes remained almost constant till day 7 (**Figure 2-7.C**).

2.3 Temperature affects crosslinking efficiency

As described earlier (section 2.1.2), overheating of the chip was observed. To ensure that the “crosslinking box” prevented drastic temperature variations on the chip, a sensor was inserted between the PDMS layer and the glass slide covering the channels. It was observed that for a period of 15 minutes (general duration of one experimental run), the temperature of the chip remained well below 35°C (close to the normal body temperature of humans) even at the highest irradiance of the CL₃₈₅ lamp (**Figure 2-8.A**). However, while resetting the chip for a second experimental run, the serpentine channel part of the chip felt hotter. To understand the reason behind this, another measurement was done keeping the sensor exactly at the centre of the serpentine channels in line with the illumination spot from the LED lamp. This experiment turned out to be very surprising as the temperatures rose to above 50°C within a minute for all irradiance conditions above 726 mW/cm², reaching up to around 70-80°C for the highest irradiances (1344 and 1537 mW/cm²) (**Figure 2-8.B**).

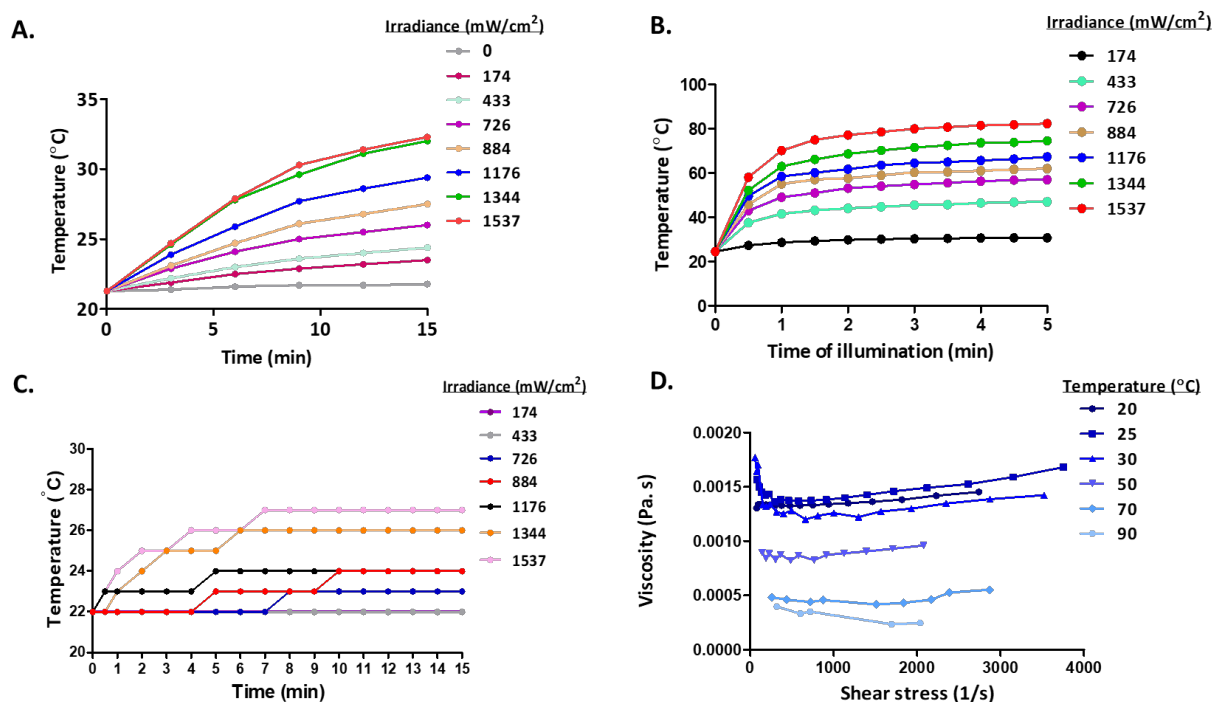


Figure 2-8: Temperature conditions within the system. **A.** Temperature of CC (not at the illumination spot), and **B.** temperature at the illumination spot on the CC for all irradiances of CL₃₈₅. **C.** Temperature of the oil flowing out of the CC from the outlet after being exposed to the CL₃₈₅ irradiances. **D.** Rheological measurements of hydrogel in oil for temperatures similar to those experienced in the OTF system during the experiment.

The results from the illumination spot experiment were greatly concerning, and hence the first experiment that was performed was to check how this temperature affected the droplets flowing in oil-surfactant through this spot. Surprisingly, the oil temperature measured at the outlet after the crosslinking process showed temperatures lower than 30°C, thereby showing that the short period during which the oil passed through the illumination spot (~15 seconds) was not sufficient to heat the oil-surfactant within the channels (**Figure 2-8.C**).

A rheological analysis of the hydrogel in oil-surfactant was also performed at temperatures correlating to the illumination spot temperatures (20, 25, 30, 50, 70, and 90 °C). The viscosities of the hydrogel kept getting lower with increasing temperature (**Figure 2-8.D**). Above 70°C, the viscosities became almost three times lower than the hydrogel at 30°C. This result showed some connections to the phenomenon of droplet disappearance after 72 hours of crosslinking with irradiances at and above 1344 mW/cm². This section showed that temperature can have a significant effect on the crosslinking efficiency of the droplets.

2.4 Effect of crosslinking efficiency on cell viability

2.4.1 Irradiance affects cell viability

The droplets with cells encapsulated inside were crosslinked with CL₃₈₅ and checked for cell viability post-crosslinking and culture. The cell viability observed on Day 1 of culture showed that irradiances lower than 1000 mW/cm² were very good for cell viability (**Figure 2-9.B**). For higher irradiances, the observed cell viability was significantly lower (<70%) and kept getting worse as irradiance was increased.

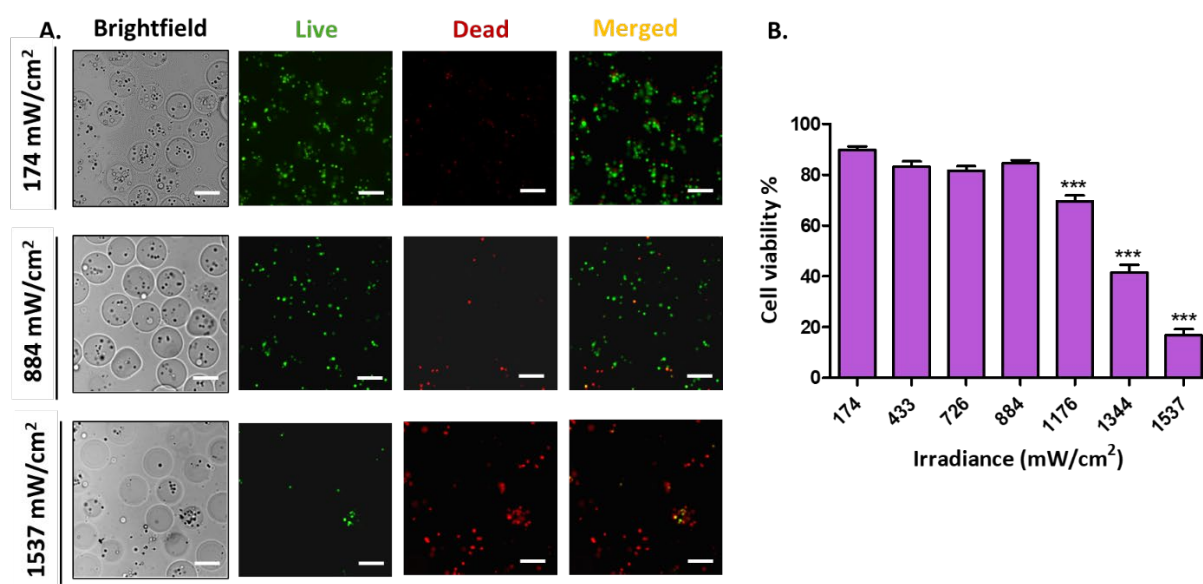


Figure 2-9: Irradiance and crosslinking efficiency affect cell viability. **A.** Viable and dead cells observed in droplets crosslinked at lowest, optimum, and highest irradiances (from top to bottom) for the CL₃₈₅ lamp (Scale bar = 200 μ m). **B.** Cell viability percentages were observed for each irradiance interval.

For choosing the optimum conditions for future tests, a balanced approach was considered. Since the droplets needed to have good stability to facilitate long-term cultures, they needed to be stably crosslinked while maintaining good cell viability. At the best crosslinking condition (884 mW/cm²), the cell viability was found to be sufficiently high (84.5%±1.3%) and hence was selected for all future experiments.

2.4.2 Near-UV wavelength also supports high cell viability

According to some previous research, visible light is better for photo-crosslinking applications, with lesser cytotoxicity compared to UV⁶⁹. To ensure that our choice of lamp (CL₃₈₅ over CL₄₀₅) does not significantly affect the cell viability of encapsulated cells, the live-dead test was done for droplets crosslinked using 884 mW/cm² using both the CLs (**Figure 2-10.A**).

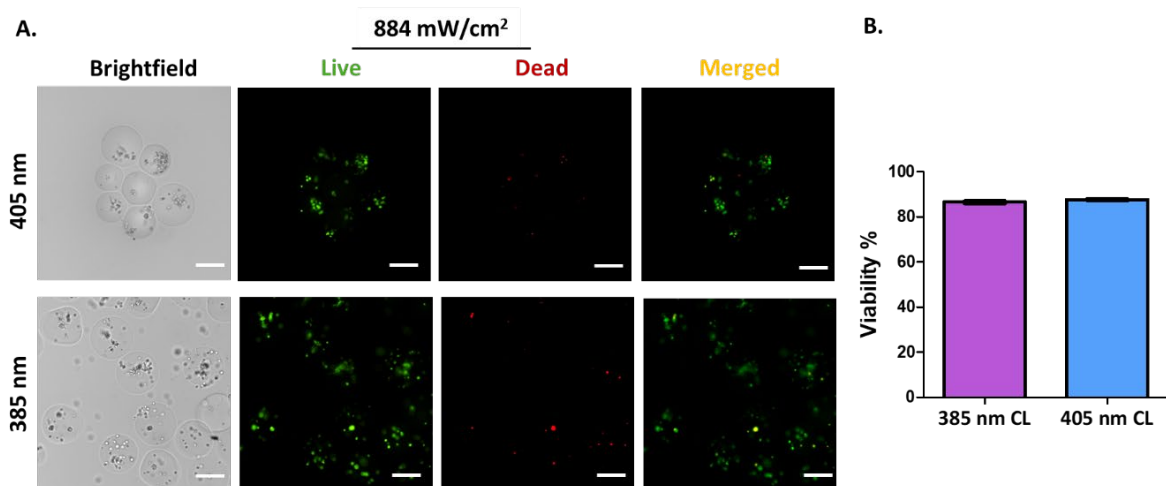


Figure 2-10: Effect of crosslinking light wavelength on cell viability. **A.** Dead(red) and live (green) cells observed in droplets crosslinked at 884 mW/cm² on day 3 of culture for both CLs (Scale bar = 200 μ m). **B.** Cell viability percentages in droplets compared between the two wavelengths.

In both cases, cell viability was found to be comparable, with an average of just around 1% higher viability for the CL₄₀₅ crosslinked samples compared to the CL₃₈₅ crosslinked samples at Day 3 of culture. The values were not significantly different, and hence the CL₃₈₅ could be safely used for our applications for developing artificial islets. However, sensitivity to UV light might vary depending on the sensitivity of a particular cell type. The cell susceptibility to the two wavelengths was checked later for pancreatic islet cells as well.

2.5 Effect of photo-crosslinking on droplet structures

2.5.1 Droplet diameter changes during different stages of the droplet formation process

Based on the optimizations performed earlier, a complete experiment was performed using the OTF crosslinking system. The flow rates were maintained at 25-125 μ L/min (Q_H-Q_O), and a cell concentration of 20 million cells/mL was used for the experiment. Crosslinking was performed at 884 mW/cm² irradiance using the CL₃₈₅ lamp. Droplet diameters were calculated for all important stages of formulation (before and after crosslinking and immediately, and 24 hours after washing).

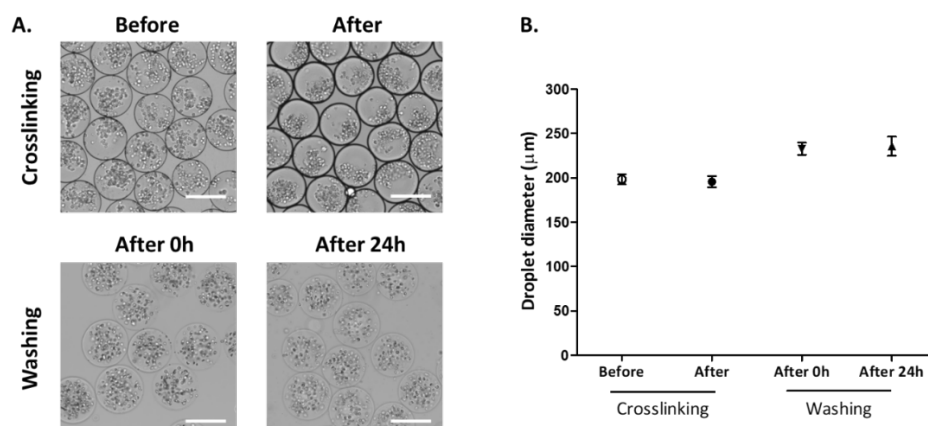


Figure 2-11: Droplet sizes at different stages of the formulation process. **A.** Phase-contrast microscope images showing cell-laden droplets at different stages of the process (Scale bar = 200 μ m). **B.** Average droplet diameters measured for droplets at each stage.

The droplets produced from the DPC were collected and had an average diameter of around $198\ \mu\text{m}$ ($\pm 5.7\mu\text{m}$). After crosslinking on the OTF system, the droplet diameters did not show any significant changes ($195\ \mu\text{m} \pm 6.3\mu\text{m}$). Immediately after washing, the average droplet diameter increased to $232\ \mu\text{m}$ ($\pm 6.9\mu\text{m}$) due to swelling of the hydrogel. After 24 hours, the average droplet size just increased to around $235\ \mu\text{m}$ ($\pm 10\ \mu\text{m}$), indicating no significant size increases due to swelling (**Figure 2-11**). The swelling of the droplets without any signs of dissolution after 24 hours indicated that they were well-crosslinked and stable.

2.5.2 Features observed in partially crosslinked droplets

It was observed previously that a “shell” of partially crosslinked hydrogel formed due to insufficient irradiance or crosslinking time. On careful inspection, the shells could be seen as a concentric layer prior to washing (**Figure 2-12.A**). After washing, the “shell” was clearly distinguishable from the “core”. A single droplet boundary was observed for the optimum condition ($884\ \text{mW}/\text{cm}^2$) before washing and formed a very small “shell”-like structure after washing (**Figure 2-12.B**).

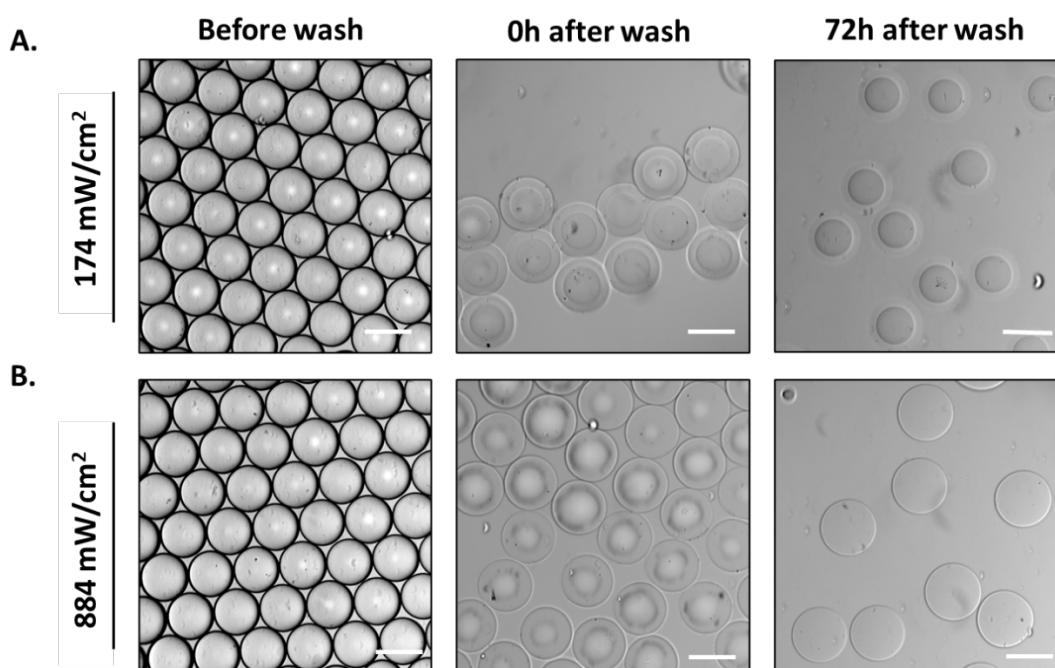


Figure 2-12: Features formed during crosslinking. **A.** Cap-like structures seen for droplets in oil when incompletely crosslinked, the presence of “shell” in such droplets after washing, and their (shell) dissolution over 72 hours in culture. **B.** Uniform circular droplets without caps in oil (left) and formation of a thin shell ($\sim 10\ \mu\text{m}$) after washing in these droplets and retention of their stable structures after 72 hours.

After washing, the droplets crosslinked at a lower irradiance had a bigger shell as expected. Over 72 hours in culture, the shell dissolved for the lower irradiance droplets, proving that the crosslinking of the “shell” was unstable. The “core” of the droplets, however, retained its original size. For the optimum condition of irradiance, the droplet structure remained the same even after 72 hours in culture, indicating that its “shell”-like feature was not formed due to incomplete crosslinking and remained as a stable droplet structure. Droplet diameter increased due to swelling of the hydrogel in the case of the optimally crosslinked droplets.

2.6 Increasing production rate of the OTF system

It has been demonstrated earlier that the DPC has the capability of achieving ultra-high throughputs in the order of up to 10^5 droplets /minute (Chapter 1, Section 2.3.1, Results). For the OTF system, an increase in production rates was attempted. Two flow rates were selected Q_H - Q_O of 50-100 $\mu\text{L}/\text{min}$ and 150-300 $\mu\text{L}/\text{min}$. In the first case, the total flow rate was maintained (total 150 $\mu\text{L}/\text{min}$), but the hydrogel flow rate was doubled to double the number of droplets produced per minute. The same CC design (termed CC-A in **Figure 2-13**) was used for this case. For the second case, the net flow rate was three times higher than the previous case (450 $\mu\text{L}/\text{min}$). The Q_H was 6 times higher for this case. To accommodate the required 15 seconds of exposure for crosslinking, another version of the CC channel with 3 times higher channel length (with 39 turns, termed as CC-B in **Figure 2-13**) was used for this experiment.

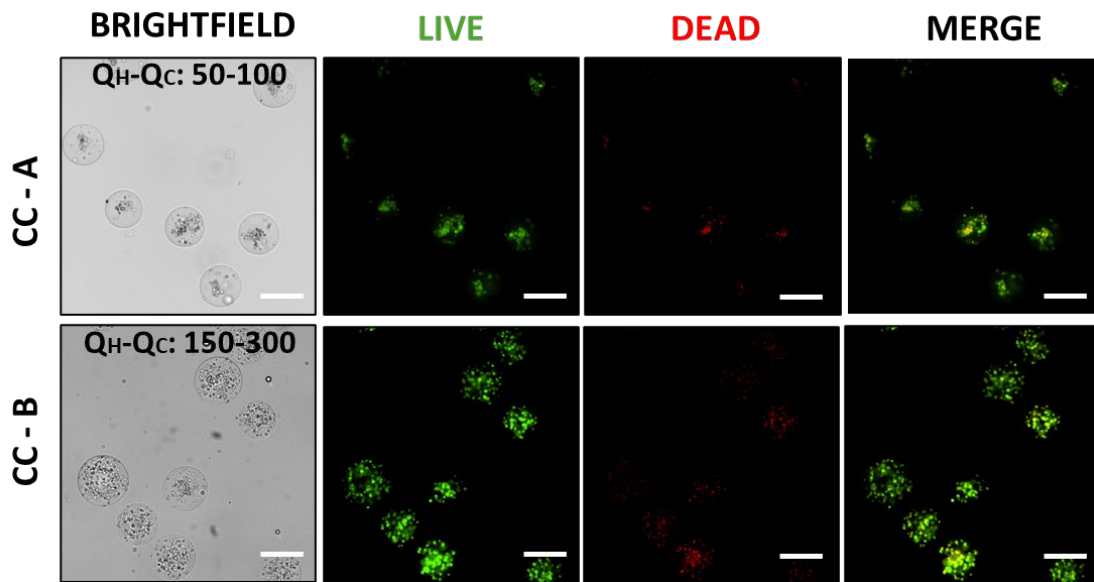


Figure 2-13: Increasing droplet production rate on the OTF system. For two times and six times higher hydrogel flow rates, no significant difference in viability was observed (Scale bar = 200 μm).

Cell viability in both cases was found to be around 70% ($\sim 68\%$ and $\sim 71\%$ in the first and second cases, respectively) after Day 1 of culture. The cell viability in the optimal case was higher ($\sim 80\%$), possibly due to lower viscous stresses due to the higher continuous phase volume fraction and lower hydrogel flow rate. The production rate, however, could be increased to ~ 16000 droplets/min and ~ 50000 droplets/min compared to the ~ 8000 droplets/min using the optimum conditions with the OTF crosslinking system without significantly affecting encapsulated cell viability. For the islet experiments, however, the optimal flow rate (25-125 $\mu\text{L}/\text{min}$) was used to obtain the maximum cell viability possible with this system.

2.7 Stationary vs. on-the-crosslinking: benefits and problems

To design the OTF crosslinking system, the hydrogel bulk samples were always checked by stationary crosslinking methods to get an estimate of the time required to crosslink the

hydrogel. The equivalent exposure time was used to determine the flow rates required for the OTF system. In some cases (for sensitive cells), however, the droplets were crosslinked using the same stationary system (described in Section 1.2 of Materials and Methods). Based on our observations, we found the following benefits and limitations of each of the systems:

OTF crosslinking system:

Benefits:

- Uniformly crosslinked beads obtained with high spatio-temporal control over each droplet during the crosslinking process as they flow in a single layer through the same material and distance (**Figure 2-14.A**).
- Automated, robust process for obtaining crosslinked droplets in high-throughput.
- Minimal interference of human handling, so it is highly reproducible.

Limitations:

- Flow rate-based time-dependent process. For faster production, factors such as CC design, lamp power/irradiance, or hydrogel properties need to be altered, which require long optimization periods.
- Cannot be performed for cell lines sensitive to fluidic or viscous stress factors.
- Multiple tubing connections and interconnected parts are present. If there is a problem with any part of the system, the whole system gets affected (especially clogging issues).

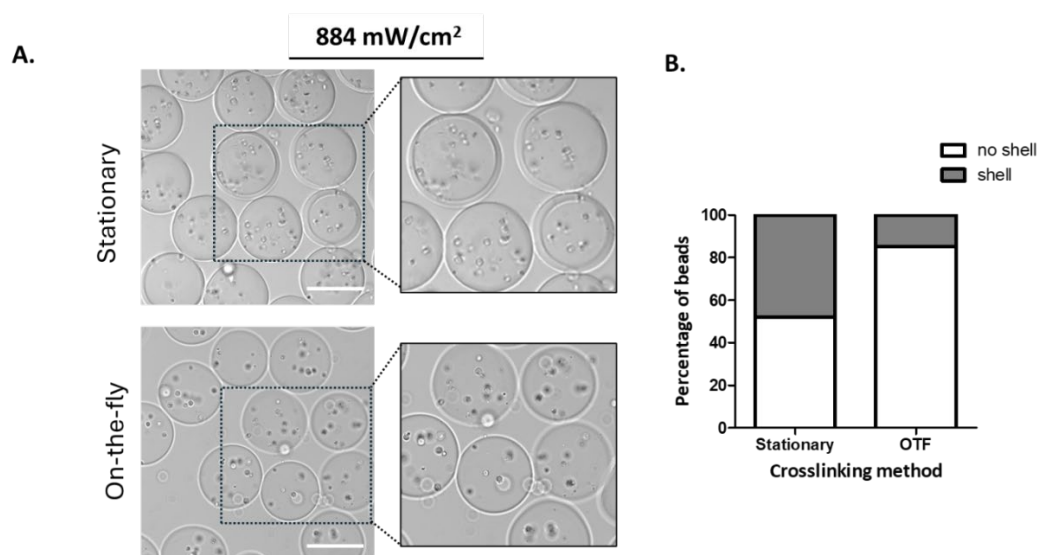


Figure 2-14: Stationary vs OTF crosslinking based on droplet appearance. **A.** Phase-contrast microscope images showing the presence of partially-crosslinked “shells” in some of the stationary crosslinked droplets, while uniform droplet structures can be seen for OTF crosslinking (Scale bar = 200 μm). **B.** Percentage of crosslinked beads showing the presence of “partially-crosslinked “shells” for each crosslinking method.

Stationary crosslinking system:

Benefits:

- Very high droplet production rates can be used on the DPC, followed by a one-time crosslinking step for crosslinking all produced droplets at the same time.
- Simple system without interconnected parts and easy to handle.
- Can be used for all cell types (other than UV-sensitive cells) without any stress factors from the system (very low system-residence time)

Limitations:

- Poor spatio-temporal control over individual droplets leading to non-uniform structures. Uneven crosslinking of droplets based on their position in the collection container. Droplets at the edges of the collection container or in upper layers get incompletely crosslinked, leading to the formation of partially-crosslinked “shells” on them (**Figure 2-14.B**). In the OTF system, such droplets can only form in the case of clogging, which can be avoided.
- Can be affected by factors such as the collection dish material based on light absorbance/transmittance or temperature resistance.
- Sufficient volume of oil-surfactant required to prevent drying up of droplets during crosslinking.

3 Discussion

[On-the-fly crosslinking demonstrated by others]

Photo-crosslinking on-chip has been attempted by a few other researchers to achieve better crosslinking uniformity and efficiency while maintaining throughput. In a very similar work to our CC chip design, Mohamed *et al.* (2019) used a serpentine channel chip for producing crosslinked GelMA droplets with encapsulated cells at 405 nm light⁷⁰. The cells were pre-mixed with BSA to ensure better viability (around 85%). However, the crosslinking happened in a diamond-shaped chamber on their crosslinking chip, almost in a stationary manner, and then flowed out through the serpentine channels, unlike in our system, where crosslinking happened when the droplets were in flow. Akbari and colleagues demonstrated a chip that was capable of producing UV-crosslinked PEG diacrylate (PEGDA) droplets for cell encapsulation⁷¹. However, the system could not produce highly monodisperse droplets as compared to our system. These crosslinking systems were on a single chip, which acts as a limitation to using stronger light sources or higher throughput methods, as they might affect the droplet generation process. In this case, our two-chip system allows segregated functioning to allow maximum output from each part of the process. Xue and colleagues produced tumour cells encapsulated in PEGDA droplets on a T-junction chip and subsequently photocrosslinked in a serpentine channel at 125.4 mW/cm² irradiance of UV light⁷². PEGDA droplets were produced in ultrahigh throughput in another 2-chip system and photocrosslinked by varying

UV dosage⁷³. A related research produced BSA-based droplets that were photochemically crosslinked within a coiled channel in the presence of ruthenium-based radical and blue LED light (452 nm) at 40 mW/cm² for droplets produced using a flow-focusing device⁷⁴.

[On-chip crosslinking for other crosslinking types]

On-chip crosslinking of droplets by chemical methods has also been demonstrated in several studies as one of the techniques shown by Akbari *et al.* (2017) with the same device mentioned for PEGDA droplets for chemically crosslinking alginate microparticles as well⁷¹. RGD-modified alginate was used to encapsulate single MSCs and mixed with Calcium-EDTA solutions and crosslinked in continuous flow with a similar continuous phase as in our case (HFE 7100 with Krytox-PEG surfactant)⁷⁵. Alginate droplets with encapsulated multiple bacterial cells/droplet were produced in a co-flow chip, followed by crosslinking in the CaCl₂-plug located at the T-junction of the same chip⁷⁶. An innovative active droplet generation technique was used to produce water-in-water droplets using a piezo-disc embedded device⁷⁷. Crosslinking of these methacrylated dextran droplets (with TEMED added for speeding crosslinking) was done in flow with a PEG-rich aqueous stream with the initiator ammonium peroxydisulfate.

[Stationary crosslinking systems for hydrogel crosslinking for 3D cell culture]

Most of the stationary phase crosslinking was done using photomasks or photopatterning techniques⁷⁸. A photopatterned PDMS-based chip was used to obtain GelMA and GelSH (thiol-modified gelatin) 3D structures on-chip for organ-on-chip applications. The hydrogels in this system were crosslinked with 405 nm light, and high cell concentrations (>10⁷ cells/mL) were attempted for encapsulation⁷⁹. Such methods, however, have a monolayer of droplets where the crosslinking efficiency remains fairly uniform, which is not possible for high-throughput applications, as in our system, where we see variations in droplet crosslinking efficiency between droplets.

[Crosslinking efficiency and factors that might affect it]

The main effect on photo-crosslinking efficiency stems from the inhibition of the radical formation step. The factor that can affect this the most is inhibition by oxygen present in the hydrogel's surroundings (in the continuous phase and diffused from PDMS layers)^{80,81}. To handle this issue, inert-gas purging, using alternate materials for chip fabrication, or altering the crosslinking chemistry are used⁸². Cell loading concentrations were found to affect the rheological features of the final gel formed in gelatin with photo-crosslinkable groups. Lower storage moduli were observed in GelMA that encapsulated $\geq 10^7$ cells/mL⁷⁹.

[Crosslinking affecting cell viability]

Among factors affecting cell viability, UV exposure time could affect it inversely, while higher UV intensities do not significantly affect viability due to different rates of the radical formation process⁸³. This is probably why large numbers of apoptotic/necrotic cells were observed during initial optimizations, where flow rates were low but quite low UV irradiances were used. As for high irradiances, up to 884 mW/cm², cell viability was found to be quite high. Higher irradiances showed a decrease in cell viability, but an associated drastic temperature rise at such irradiances was observed, which might be a cause of this cell death⁸⁴. Higher pre-polymer concentrations were found to cause lower cell viability, similar to our observations⁸⁵.

[Application tested: Long-term 3D culture]

The crosslinked droplets developed using the OTF system during this project were first tested for their stability in culture, which is a necessary parameter for cell therapy strategies. Firstly, L929 fibroblasts encapsulated in GelMA droplets were checked for cell viability and proliferation over a 2-week period (**Figure 2-15**). The droplet maintained its structure over the two weeks. The cells proliferated rapidly as in 2D culture, and after 5 days, they started migrating out of the droplet and growing on the droplet surface instead, resulting in microtissue formation.

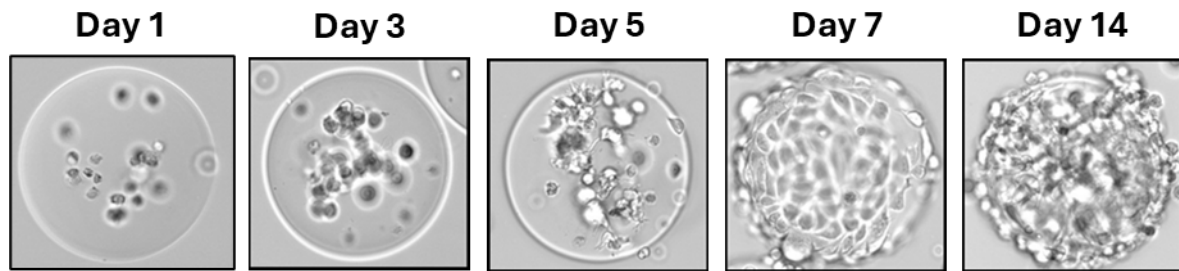


Figure 2-15: Long-term culture of L929 fibroblasts encapsulated in crosslinked GelMA droplets showing cell proliferation by Day 3, migration of cells to the droplet surface by Day 5 and microtissue formation on the droplet by Day 14.

[Possible future work]

Alterations can be made to make the crosslinking process more efficient by using alternate materials for the CC fabrication or by adding chemical factors into the system to inhibit the effects of radical neutralizing elements, to allow better crosslinking. The system can be improved to allow uniform flow throughout the system without the formation of clogs, which is a common problem in the current system. Miniaturization of the system can be attempted in the future as well, with a possible integration of a simple washing process integrated to the OTF system.

Chapter 3 : Hydrogel Optimizations for 3D-Cell Culture Applications

Cell culture in 3D has been recently gaining massive popularity due to their high physiological relevance compared to the existing 2D culture methods. For such 3D applications techniques such as bioprinting, on-chip patterning, and microfluidic generation have been widely used. For such applications, porous soft-materials such as hydrogels have become the material of choice due to their ease of production and biomimetic features.

Hydrogels: Basic properties

Hydrogels are polymeric materials containing a large proportion of water as the dispersion medium. In general, a gel is a type of colloidal mixture that has microscopic particles (ranging from 1-1000 nm in diameter) evenly dispersed within a continuous phase (similar to a solvent in a solution)^{86,87}. The process of forming gels involves converting the liquid mixture into a viscous state such that it behaves similarly to a solid. Other types of gels that are used commonly include organogels (organic phase trapped in 3D crosslinked structure), xerogels (solid gels with large surface area ($>150 \text{ m}^2/\text{g}$) and high porosity (up to 50%)), and aerogels (ultralight structures with low density and thermal conductivity produced by replacing gases as the continuous phase)⁸⁸.

The hydrogels are hydrophilic biomaterials and can maintain their 3-dimensional porous structure and swell in water. Their porous structure facilitates the exchange of materials such as solutes, biomolecules, gases, nutrients, and wastes. This property is necessary for cells to grow, proliferate, and function properly. The term “hydrogel” was first used by Jakob Maarten van Bemmelen, a professor of Chemistry from Leiden University, in 1894⁸⁹. Among biomaterials used for replacement in the human body, hydrogels were the first to be approved due to their low immunogenicity and great biocompatibility. The use of hydrogels for biological applications was first reported by Wichterle and Lím in their article in 1960⁹⁰. They discussed their hydrogel, poly (2-hydroxyethyl methacrylate) (pHEMA), and described its use in contact lenses. Till the present time, this hydrogel has been used in biomedical applications such as wound dressing, tissue regeneration, drug delivery, artificial skin, artificial cornea, and several others⁹¹.

Classification of Hydrogel Types

Hydrogels can be categorized based on various factors such as their source, composition, crosslinking method, and electric charge. The hydrogels can be of different types based on the source material:

- **Natural:** These hydrogels are produced using macro-biomolecules present in living beings. Mainly, polysaccharide-based and protein-based hydrogels are in this category. Some examples of natural polymers include hydrogels derived from chitosan, alginate, dextran, cellulose, whey protein-derived hydrogels, and methacrylated proteins like gelatin, collagen, and others. Since they are derived from natural sources, these hydrogels are highly biocompatible and have synergistic effects. However, some

disadvantages of such hydrogels are the variability in chemical properties depending on the different sources from which such proteins or polysaccharides are extracted.

- Synthetic: To have consistent chemical and physical properties, synthetic hydrogels are produced. However, many of these hydrogels lack biological activity or cell-binding sites and even cause toxicity, thereby limiting their application in cell/tissue culture. Some examples of such polymers include polyvinyl alcohol (PVA), polyethylene glycol (PEG), Polyacrylamide (PAAm), and so on.

Hydrogels can be further classified based on their composition, including different biomolecules (combination hydrogels, e.g., interpenetrating polymeric hydrogels), complex monomers (e.g., block copolymers), or a single pure monomeric unit (homopolymeric hydrogels).

On the basis of the ionic charge carried by these hydrogels, they can be also classified into non-ionic (neutral) e.g., pAAm, PEG, pHEMA; ionic (anionic-negatively charged; cationic-positively charged) e.g., poly vinyl pyridine, aminoethyl methacrylate (AEMA); zwitterionic (containing both cationic and anionic groups) e.g., polybetaines and polyampholytes; and amphoteric (containing both acidic and basic groups) e.g., N-isopropylacrylamide/[{3-(methacryloyl-amino) propyl}dimethyl (3-sulfopropyl) ammonium-hydroxide]- (NIPAAm/MPSA)⁹².

Use of methacrylated hydrogels for cell culture

Hydrogels are widely used as scaffolds for 3D cell culture. Depending on the porosity and stiffness of the material, the effect of different hydrogels on cell growth, proliferation, and functionality can vary. The hydrogels can act as an alternate extracellular matrix (ECM), providing the cells encapsulated inside with important growth factors and a surface to bind to (not naturally present in synthetic hydrogels). At the pre-polymer state, these hydrogels are in a liquid state, which enables them to be used easily for injectable therapies or in microfluidic-assisted fabrication/printing applications. A wide variety of techniques using light (photo-crosslinking), temperature, pH, or chemical methods can be used to polymerize or crosslink these hydrogels to form stable gels with great structural integrity. These structures allow cell entrapment while the pores in the structure allow the exchange of nutrients, gases, and cellular secretions and wastes, making these hydrogels very useful for tissue engineering applications.

Characteristics of natural hydrogels: GelMA and HAMA

For this project, we have used natural hydrogels made from gelatin and, in some cases, with added hyaluronic acid or organ-derived decellularized ECM. The gelatin and hyaluronic acid were converted to photocrosslinkable hydrogels by methacrylation.

Gelatin is a protein derived from collagen abundantly present in animal skin, bones, and organs. It retains the tropocollagen rod from the triple helical structure of collagen. Unlike collagen, gelatin has a linear RGD (R-arginine, G-glycine, D-aspartate) motif available in its β -subunit I-like-domain for cell adhesion using integrins (especially $\alpha_5\beta_1$ and $\alpha_v\beta_3$). In the presence of divalent ions like Mg^{2+} , these integrins present in cells ligate to the RGD⁹³.

Gelatin, in its natural form, can gelate at temperatures lower than 25°C; however, at physiological temperatures (~37°C, commonly used for mammalian cell culture), it liquifies.

Therefore, to make the hydrogels stable at higher temperatures, chemical or photo-crosslinking was used by modifying some of the several side chains present in their chemical structure. The gelatin (also for hyaluronic acid) hydrogels were obtained by methacrylation to allow photo-crosslinking with UV light or γ -rays in the presence of a photoinitiator. The methacrylated gelatin (GelMA) hydrogels can be easily produced by reacting methacrylate anhydride with gelatin to replace the primary amine groups (**Figure 3-1**; detailed protocol in Section 1.1)⁹⁴. A similar strategy is followed to produce methacrylated hyaluronic acid (HAMA). The first UV-crosslinkable GelMA was reported in 2000 by Van den Bulcke and colleagues⁹⁵.

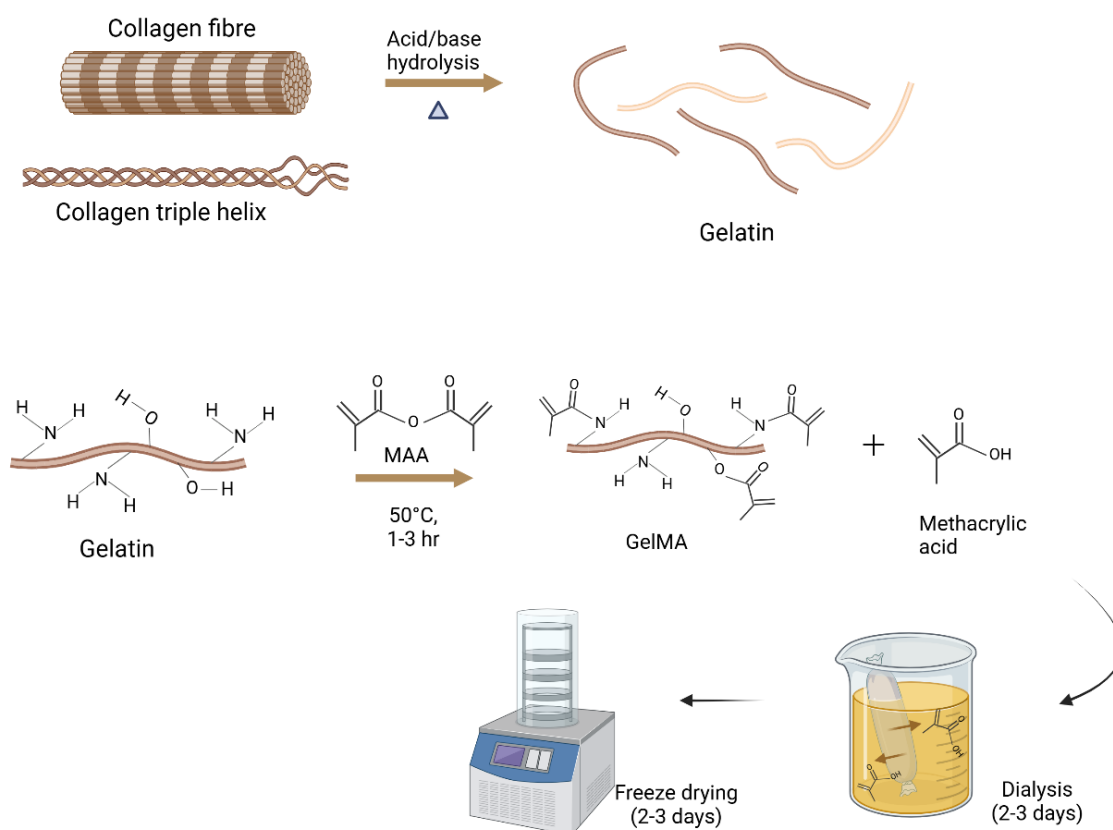


Figure 3-1: Complete production procedure of methacrylated gelatin (Created in <https://BioRender.com>).

Hyaluronic acid (HA) is a polysaccharide commonly found in the ECM throughout the body, in cartilage tissues, and in the vitreous region of the eye⁹⁶. It consists of repeating units of β -1,4-D-glucuronic acid and β -1,3-N-acetyl-D-glucosamine to form an alternating copolymer. HA serves different roles in the body, such as promotion of chondrogenesis, cell signalling, wound healing, and matrix remodelling⁹⁷. It can provide high degrees of mechanical strength in hydrogels. The first methacrylation of HA was demonstrated by Smeds and colleagues in 1999⁹⁸.

Role of decellularized ECM for Cell Culture

Extracellular matrix (ECM) is a substrate for cells to attach to for growth and proliferation. The ECM plays an important role in providing growth factors to cells, allows cellular crosstalk, aids in differentiation, and helps sustain cell morphology and physiology⁹⁹. The composition of ECM varies between different tissues and organs and includes a large number of proteins

and certain carbohydrates¹⁰⁰. Due to such structural complexity, it is difficult to produce such ECM matrices by conventional methods, which is why it is derived from originally present ECM from organs or tissues (or even cells) by a process called decellularization and reconstituted. Such decellularized ECM (dECM) can be used for various tissue/organ engineering applications on their own or in addition to hydrogels to mimic the native microenvironment for the cells¹⁰¹.

In this chapter, we investigated different hydrogel compositions to find the hydrogels most suitable for our artificial islet models. Firstly, we tried to develop a PDMS-based device with wells for screening dECM-containing bioinks. Several surface modifications were tried to convert the hydrophobic PDMS surface into a hydrophilic one to enable the spreading of the hydrogels on the well surface for even crosslinking. Alternatively, hydrogels were tested in commercial treated polystyrene well plates to obtain the best hydrogel for cell culture based on cell viability, proliferation, and hydrogel degradability. Selected hydrogels were used for droplet fabrication with different cell types encapsulated in them. Optimizations were made to determine the perfect composition with GelMA, HAMA, and dECM from the porcine pancreas to obtain the best hydrogel mixture for high growth, proliferation, and tissue-forming characteristics in encapsulated cells in durable droplets.

1 Materials and Methods

1.1 GelMA synthesis

Different methods were tried for GelMA preparation. Firstly, PBS buffer (400 mL) was used to dissolve 10 g of gelatin (Type A, Bloom 300; Sigma-Aldrich) completely using a magnetic stirrer at 60°C. To this solution, 8 mL of methacrylic anhydride was added dropwise while stirring. The reaction was allowed to be carried on for 3 hours, after which two volumes of preheated PBS were added to stop the reaction¹⁰².

The other method for GelMA synthesis used for a better degree of methacrylation was done using carbonate-bicarbonate buffer at pH 9 according to the protocol described by Shirahama *et al.*, 2016¹⁰³. A 0.25M concentration of the buffer was prepared with 26.49 g/L Sodium carbonate and 21 g/L Sodium bicarbonate solutions in proportion to give a pH of 9. The gelatin was dissolved as described before in this CB buffer at 50°C and stirred at 300 rpm. To the gelatin solution, 0.1 mL (per gram of gelatin) of methacrylic anhydride was added, and the pH was adjusted to 9 with 6M HCl or 6M NaOH. The reaction was allowed to proceed for 1 hour and stopped by adjusting the pH to 7.4. A general schematic of these methods has been shown in the introduction of this chapter (**Figure 3-1**).

1.2 Hydrogel preparation for testing

Bioinks and their components (GelMA, HAMA, and porcine pancreatic dECM) discussed in this chapter and henceforth were provided by our collaborators, Fundacja Badań i Rozwoju Nauki (FBiRN), Poland, for this project (or purchased from their start-up, Polbionica).

For testing, the freeze-dried GelMA or HAMA or both were dissolved in PBS in the required proportions by stirring at 400 rpm for 1 hour at 50°C. The hydrogel solution was filtered using a 0.22 µm syringe filter (Millipore GmbH, Germany), and the photoinitiator was added to obtain a final concentration of 0.4% in the hydrogel solution.

1.3 Hydrogel Optimization Chip

To test different hydrogel compositions in bulk (10 µL drop), an 8-well chip was fabricated. A 3D-printed mould was made with 8 raised, interspersed cylindrical protrusions. These moulds were used for making PDMS chambers with 8 wells, designed in a way that each of these wells receives an equal amount of light during crosslinking. The wells had a 200 µm diameter (to mimic our droplet diameters) and 200 µm step height to accommodate exactly 5 µL of hydrogel pre-polymer solution. For ease of microscopic analysis, these chips could be directly bonded to glass slides during fabrication (when the PDMS is in liquid form). With multiple 3D printed moulds, the chips could be produced on a large scale (up to 10 chips/day) for single-use, cell-based testing. Alternatively, a standard commercially available polystyrene 24 or 96-well plate (for viability tests) was used for such hydrogel optimization tests.

1.4 Chip Surface Modification

Several different surface modification techniques were tried to convert the hydrophobic PDMS surface into a hydrophilic one so as to enable even spreading of the hydrogel material in the well. Firstly, the surface modifications were tried on thin PDMS films.

The physical surface technique tried was long exposure (1-3 min) to oxygen plasma at high intensity. The addition of water or ethanol to the wells was tried to stabilise the hydrophilicity.

For chemical surface treatments, several strategies were tried. Firstly, a combination of hydrogen peroxide, hydrochloric acid and water in equal proportions was used to pre-coat the surface. After 30 mins, this solution was washed out with distilled water, the wells were air-dried, followed by the addition of APTES ((3-Aminopropyl)triethoxysilane solution (5%, 10%, and 50% in water) for another half an hour. After washing, these chips were dried and stored.

Other chemical techniques include treatment with commercial hydrophilizing solutions such as P100 and Aculon. Some polymeric coatings, such as PVA (polyvinyl alcohol) and PVP (polyvinylpyrrolidone), were tried to improve the hydrophilicity of PDMS.

Some biopolymer-based coatings were tried to improve the hydrophilicity of the PDMS and to improve protein attachment to the surface. Gelatin (1%), itaconic acid (ITA), and Gelatin-tyramine conjugate (GTA) were used for the modification.

1.5 Hydrophilicity analysis through contact angle measurement

To test the efficiency of each of the above-described techniques for inducing hydrophilicity, thin PDMS films were bonded to 0.5 mm square glass cover slips. The different surface modifications were attempted on different film surfaces and dried thoroughly. The film structures were then placed on a stage placed in line with a high-resolution optical lens (Zeiss, Germany) fitted to a uEye camera. A cemented Hamilton syringe was used to dispense exactly

2 μ L of water on the surface of the treated/untreated PDMS film to form a droplet. The height and planarity of the stage were adjusted to observe the clear boundaries of the droplet, and images were captured. The droplet edges were analysed using the ImageJ software with the plugin for contact angle measurements.

1.6 Experiment design and Biocompatibility analysis

Depending on the test substrate (regular well plate or the designed chip), 10 or 5 μ L of hydrogel mixed with cells in a concentration of 10-20 million/mL was added to the bottom of the substrate. The bulk drops with cells were crosslinked by placing the plate/chip on the CML (the first lamp to be tested). Media was added up to 100 μ L in each well (for 24-well plates, 500 μ L was added).

The biocompatibility analysis of the cells inside the crosslinked bulk was performed by two methods: (i) the Live/dead cell viability analysis kit (described earlier in Chapter 2) and observed the live and dead cells with green and red fluorescence respectively using a fluorescent microscope, and (ii) the Cell counting kit (CKK-8) by adding 10 μ L of the reagent to 100 μ L of the media, incubating for 3-4 hours and then absorbance measured at 450 nm using a UV-spectrophotometer (BioTek Synergy HTX, Agilent technologies, US). The viability of the cells was calculated using the formula:

$$Viability \% = \frac{Absorbance_{sample} - Absorbance_{Blank}}{Absorbance_{Control} - Absorbance_{Blank}} \times 100$$

1.7 Droplet preparation with encapsulated cells

Droplet preparation was initially attempted with the CML using the OTF system. Eventually, after the optimisations for all the droplet formation and crosslinking parameters were done, the optimized conditions were used for the final droplet preparations with different hydrogel compositions.

1.8 Image analysis and graphical representation

All images were analysed using the ImageJ software, and graphs were plotted using the GraphPad (Prism 5) software. Statistical analysis was done based on Student's T-test or one-way ANOVA analysis, depending on the data type. Significance values are depicted as an asterisk (*) on the graphs.

2 Results and Inferences

2.1 Bioink Testing for Microfluidic- or bio-compatibility

2.1.1 Initial tests with bioinks

Bioinks were provided to us by the institute, FBiRN, who are specialist in bioprinting of pancreas and were our collaborators during this project. For initial testing, they provided us with 4 different bioinks with the following basic composition:

- Mixture in a 1:1 volume ratio of 20% GelMA and 2% HAMA, forming a final composition of 10% GelMA with 1% HAMA
- A 5% (w/v) solution of porcine pancreatic dECM [obtained by digesting dECM (p) in an acidic solution with pepsin or collagenase or both (1X = 1 mg /mL)]
- Photoinitiator LAP used (0.2%)

The 4 hydrogels varied in the enzymatic method used for obtaining the dECM from the porcine pancreas (enzymatic composition explained in **Figure 3-2.A** box). It is worth mentioning that these tests were simultaneously performed while optimising the crosslinking light parameters. For this test, the CML was the only available lamp, and it was used for initial tests. The maximum available power during this phase for the CML was 177 mW/cm² for an input current of 1.5 A (the regulator was input current-based). The hydrogels were added at 10 μ L volume to wells in a 24-well plate and crosslinked over the CML at maximum power for different time periods (30 seconds, 1, 2, and 5 minutes).

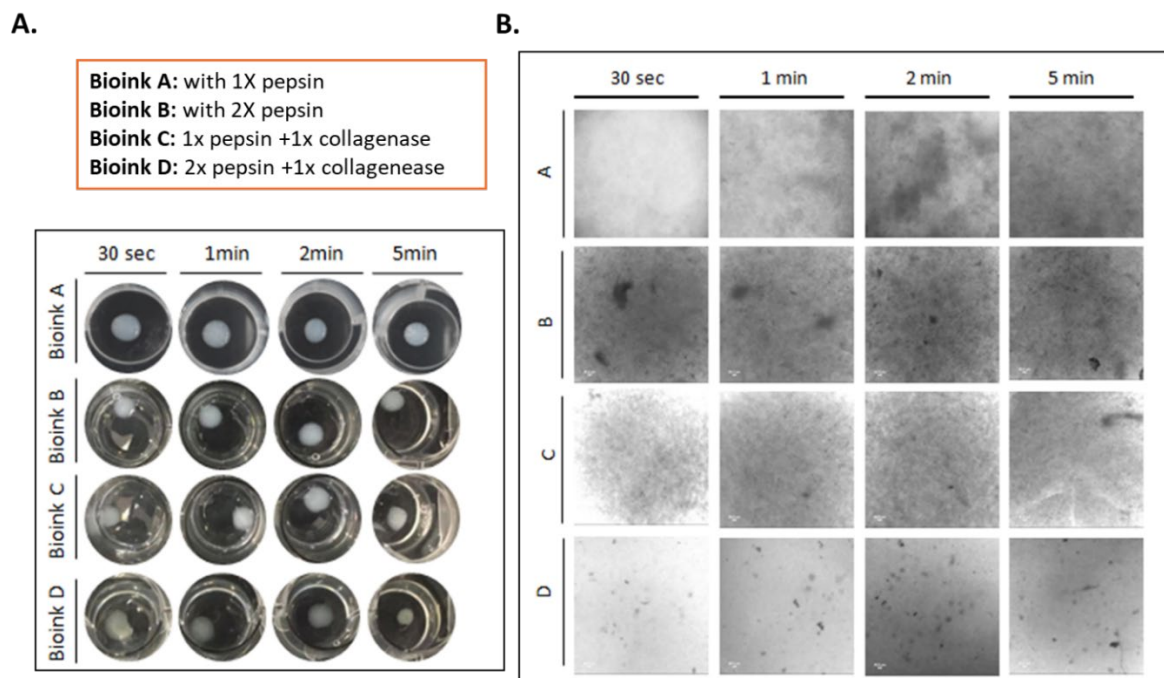


Figure 3-2: Initial bioink testing. **A.** Enzyme amount and type used to obtain the dECM for each bioink and image of the 24-well plate with the hydrogel drops for all the crosslinking conditions. **B.** Microscopic image (using 10x objective) showing the hydrogel texture for each crosslinking condition.

The hydrogels changed appearance as they got crosslinked from a cloudy or clear liquid to a dense gel. The 30-second exposure produced very soft gel or not well crosslinked gels, indicating that the time was not enough for such bioinks to get completely crosslinked. From 1 minute onwards, the gels appeared to be sufficiently crosslinked. On closer inspection under the microscope, it was found that only pepsin samples produced more visible residues on degradation than when degraded in conjunction with collagenase, where the latter produced finer residues (**Figure 3-2.B**). In terms of texture and density, the bioinks C and D appeared to have a notably better application in our microfluidic system. The bioinks A and B were too dense for use with our microchannel chips and hence were not considered for droplet formation experiments.

2.1.2 Biocompatibility analysis within the bioinks

The bioinks were mixed with the cells in a concentration of 10 million per mL in a 1:1 ratio of cells in media to the bioinks. The crosslinking was done for 1 minute for three different input current modes of the CML (0.5 A, 1.0 A, and 1.5 A) and controls without UV exposure (uncrosslinked). For the lowest irradiance conditions in the case of all bioinks, the presence of dead cells was seen at the boundary of the drop, possibly due to incomplete crosslinking (**Figure 3-3.A**). This phenomenon reduced as the irradiance was increased, and at the highest irradiance (1.5 A input current), it was not observed. The uncrosslinked controls showed mostly viable cells, indicating that the components of the bioink were not chemically toxic to the cells. Hence, the dead cells at the droplet boundaries were mostly due to free radicals from incomplete crosslinking. In the case of bioink B (the densest bioink), it was also difficult to obtain fluorescent signals from all the cells present in the bioink for uncrosslinked or partially crosslinked cases.

On closer inspection, it was noticed that not only were there red fluorescent cells indicating dead cells, but also some cells showed yellow fluorescence, which indicated penetration of the red dye into the cells, mostly due to damage to the cell membrane of the cells (**Figure 3-3.B**).

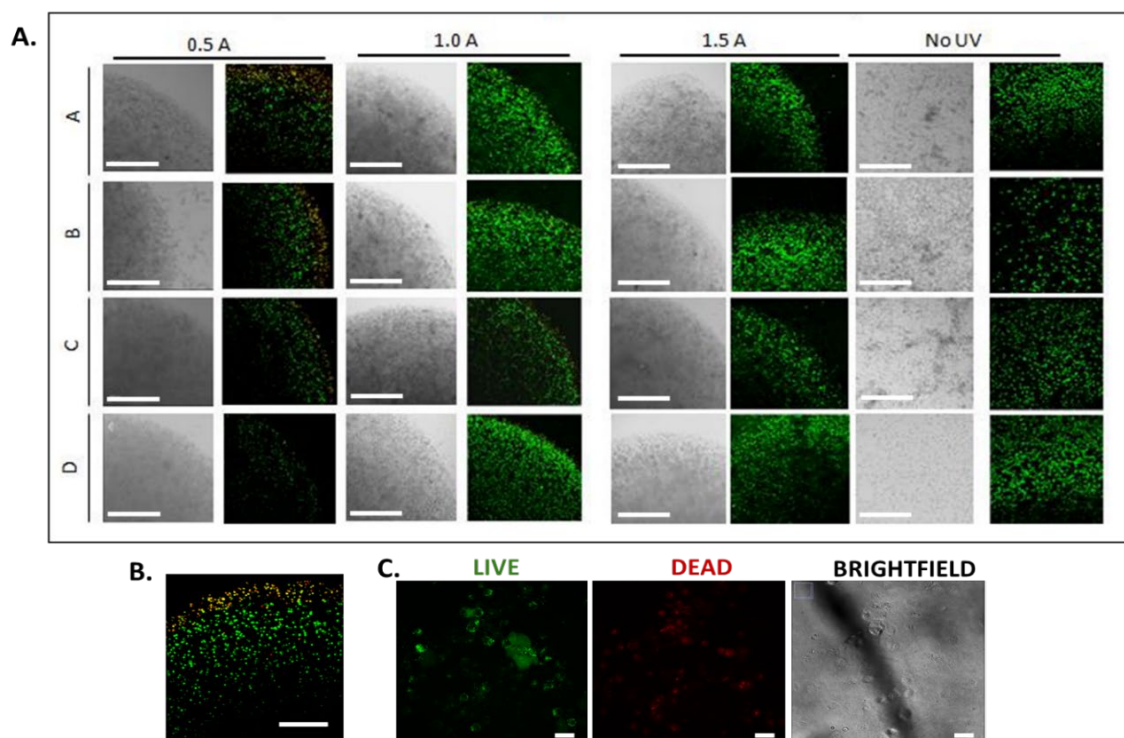


Figure 3-3: Biocompatibility analysis of bioinks. **A.** Brightfield images of crosslinked bioink drops with encapsulated L929 fibroblasts (left), with the corresponding images with the live-dead reagent with green (live cells) and red (dead cells) fluorescence. **B.** The boundary of the drop of Bioink A shows the presence of dead (red) cells or stressed cells (yellow) in encapsulation (Scale bar = 500 μ m). **C.** The dual-stained cells were analysed in detail (40x objective) to reveal a granular cell morphology with the accumulation of green dye in such granules (Scale bar = 25 μ m).

These cells were observed under the 40x objective of the fluorescent microscope to check what exactly was happening in each cell (**Figure 3-3.C**). It was noticed that the cells had a circular morphology with granules inside. The green dye (Calcein AM) appeared concentrated in these granules, while the red dye (Ethidium homodimer (Ethd-1)) also entered the cells but either fluoresced at a particular spot or remained diffused in the cell with low-intensity fluorescence. The localisation of the Calcein AM is probably due to the formation of stress granules in the cells as a result of oxidative stress from the free radicals, indicating stressed or apoptotic cells¹⁰⁴. The fact that this phenomenon happened only at the boundary region of the droplet raised the question of whether this effect would also happen if the hydrogel had equal thickness. For this purpose, a new chip was designed for bulk hydrogel testing described in the next section.

2.2 Bulk hydrogel chip development and testing

2.2.1 Chip design and fabrication

To mimic a bulk hydrogel test similar to our droplets, a chip was designed (design credits: Dr. Marcin Filipiak) with a stepped well structure. The well height was designed to be 250 μ m (similar to our droplet diameters after swelling) while the entire well was 5mm in height in the “well mould”(**Figure 3-4.A**). The well mould was milled out from a polycarbonate sheet, and the chip wall moulds (**Figure 3-4.B**) were 3D printed.

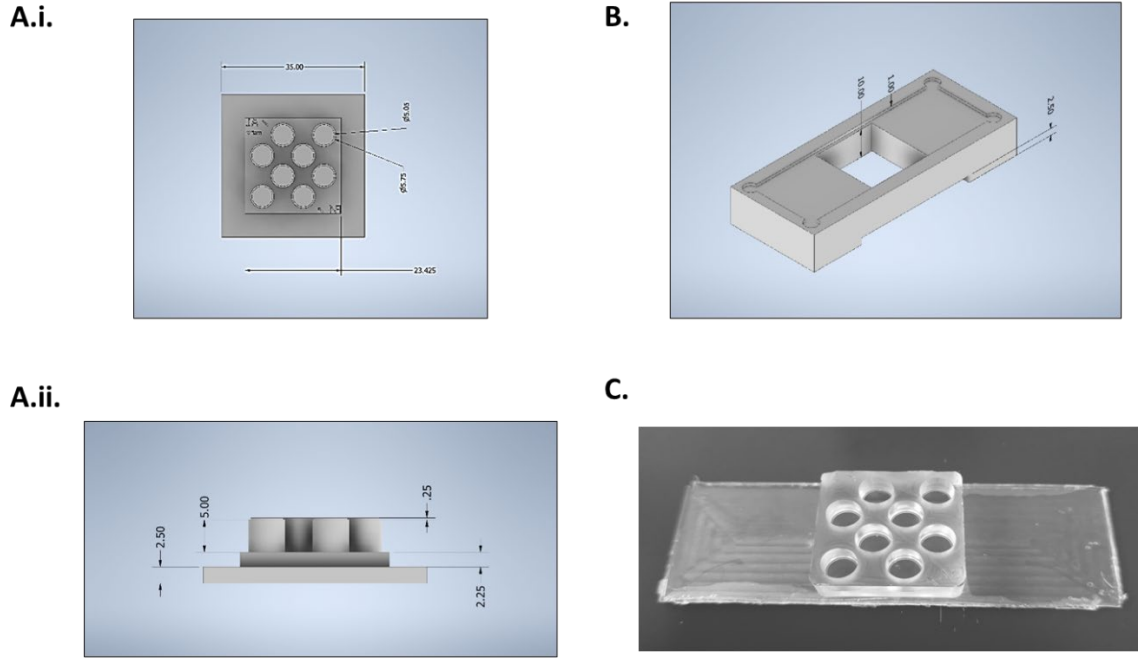


Figure 3-4: Bulk hydrogel testing chip. **A.** Mould design for the well with the stepped smaller well to accommodate the hydrogel **i.** in top view and **ii.** lateral view. **B.** The chip wall mould is to hold the liquid PDMS during fabrication and placing a glass slide on top to allow bonding of the chip to glass during PDMS curing by temperature. **C.** One of the fabricated chips using PDMS was used for the tests.

For fabrication, firstly, the well mould was fitted into the chip wall mould opening with the well bottom facing upwards (as in A.ii. orientation) and fitted perfectly (like Lego pieces). Liquid PDMS (with curing agent) was poured into the square opening over the well mould, and a glass slide of the size of the chip wall mould was slid and fixed perfectly on top. The setup was cured at 75°C for 4 hours. The final chip bonded to the glass slide (**Figure 3-4.C**) could be just pushed out of the mould. A total of 10 mould pieces (of both types) were obtained and used to make more than 100 copies of these chips.

2.2.2 Hydrophilic surface modifications for the well bottoms

Native PDMS is hydrophobic in nature, and the idea of this chip was to distribute the liquid pre-polymer hydrogel evenly at the bottom, which could only be possible if the surface was hydrophilic. While the chips were being bulk produced, thin PDMS films (of 2 mm height) were made and cut into square pieces and plasma-bonded to 0.5 mm coverslips (2.5 cm x 2.5 cm). These films were used to try out different surface treatments (details provided in Materials and Methods section, part 1.4) to convert the PDMS surface to hydrophilic (10 contact angle measurement per treatment was performed). Firstly, oxygen-plasma (O₂-plasma) treatments were attempted for 1 and 3 min (**Figure 3-5.B.i**). It was observed that immediately after plasma treatment, the surface could become sufficiently hydrophilic, but the effect was short-term (up to 6 hours). Increasing the time of treatment (up to 15 minutes) and keeping the surface submerged in water helped in obtaining lower stable final contact angle values. However, while trying the treatment on the well bottom, it was realised that the reach of the plasma to the well bottom was very poor. Hence, other chemical treatments were attempted.

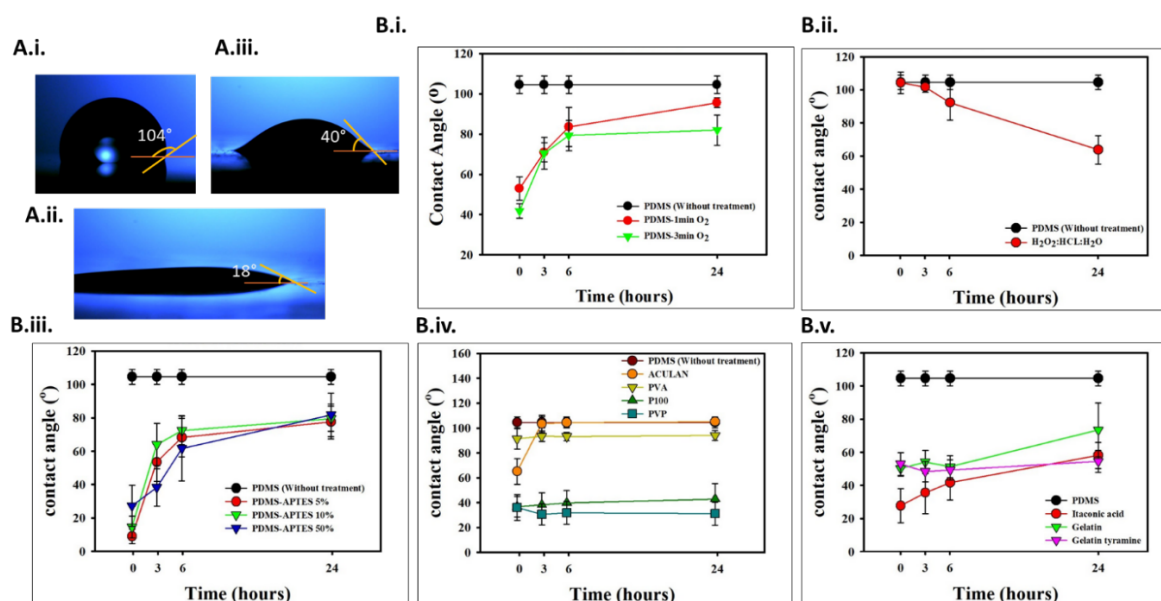


Figure 3-5: Hydrophilic surface modifications. **A.** Contact angle measurements for water on **i.** native PDMS, **ii.** immediately after modification, and **iii.** After 3 hours of modification with 50% APTES. **B.** Contact angle measurements for surface modifications with **i.** O₂-plasma, **ii.** first step of chemical modification with H₂O₂:HCl:H₂O (1:1:1), **iii.** APTES (different concentrations), **iv.** Polymers and commercial hydrophilicity-inducing solutions, and **v.** biochemical modifications.

For chemical modifications, the research papers mentioned that initial plasma treatments are necessary to activate surface groups for stabilising further surface modifications. To replace this, a chemical solution (similar to Piranha solution, composed of sulphuric acid and hydrogen peroxide) with equal proportions of H₂O₂, HCl and water was used for surface activation. This solution was found to also induce hydrophilicity on its own (**Figure 3-5.B.ii**). However, due to concerns regarding probable cytotoxicity due to the components of the solution, further modifications were attempted.

Firstly, silanization was attempted with the amine-containing silane, APTES, in different concentrations. Similar to the plasma treatments, initially the hydrophilicity of the surface was quite low (lowest values seen compared to any other surface modifications tried; **Figure 3-5: A.ii** and **B.iii**) but increased over time (**Figure 3-5.A.iii**). Another advantage of using APTES modifications is the presence of the amine groups, which is good for protein binding and could possibly help improve the adhesion of our protein-based hydrogels even after crosslinking.

Among the other treatments tried, the best results were obtained with the commercial solution, P100, and with polymer coating with PVP. These coatings induced high degrees of hydrophilicity, which were also very stable over time. For the bio-coatings, the hydrophilicity induction was found to be quite stable over time. These coatings were expected to also be good for the adhesion of our hydrogels post-crosslinking.

2.2.3 Biocompatibility of the hydrophilic surface modifications

Biocompatibility of each of the hydrophilic treatments was tested for the model cells (L929 fibroblasts) after treating the well bottom in the hydrogel testing chip. The chips were thoroughly washed, air dried, treated with UV for 1 hour and washed thrice with 70% ethanol

followed by 1X PBS in each well. Firstly, live-dead analysis was done for 10^4 cells in each well. For the cases other than the APTES and the biological coatings, cells observed on the surface were very few, as expected due to the absence of protein adherent groups on the surface (**Figure 3-6.A**). There was also a possibility that the number of cells visible on the surface was due to cytotoxicity, thereby resulting in dead cells floating in the media detached from the bottom.

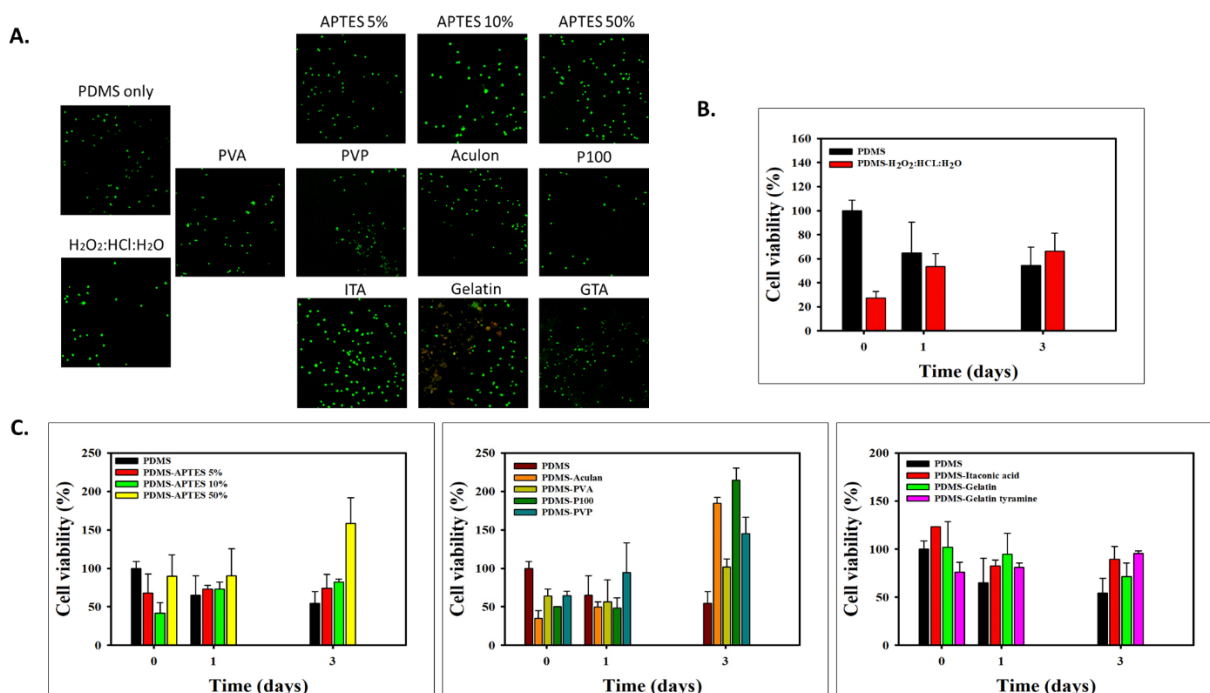


Figure 3-6: Biocompatibility for the hydrophilic surface modifications on the hydrogel testing chip. **A.** Image showing live cells attached to the bottom of the well for each surface modification method on Day 1 of culture. **B.** Cytocompatibility of native PDMS and the first surface activation stage. **C.** Percentage of viable cells observed for each modification till 3 days of culture.

To verify the cause of fewer cells on the well bottom, CCK-8 analysis was done to check for metabolically active cells. Cells in native PDMS on day 0 were considered the control sample. For the control cells in native PDMS, there was a decline in cell growth over time (a similar trend seen in gelatin-coated wells), and as expected, the activation technique led to low cell viability even though proliferation of cells happened by day 3 (**Figure 3-6.B**). Among the APTES-treated wells, 50% APTES-coated wells showed good cell viability and a good proliferation rate (even though growth was slow in the early stage). The P100 treatment had a low viability initially, but by day 3, the proliferation rate was the best among all the surface treatments (**Figure 3-6.C**). For the biological coatings, it was observed that the coating was not even on the surface, with bits and pieces of the coating in certain regions of the well (random distribution). This might be why the cell adhesion and cell viability were affected over time. Based on these results, the 50% APTES surface modification was chosen for the bioink tests.

2.2.4 Confirmation of surface modification

The surface of the treated films was analysed to check how the surface modifications affected the surface atomic composition. For this, a quantitative surface analysis technique called X-ray Photoelectron Spectroscopy was used. In this technique, a mono-energetic X-ray

is used to irradiate the surface (maximum depth of a few nanometers), resulting in the emission of photoelectrons from the surface¹⁰⁵. The energy of the emitted photoelectron is measured with the help of an analyser providing an intensity vs binding energy curve, which can be used to identify the types of atoms present on the surface, their energy state, and quantity on the surface.

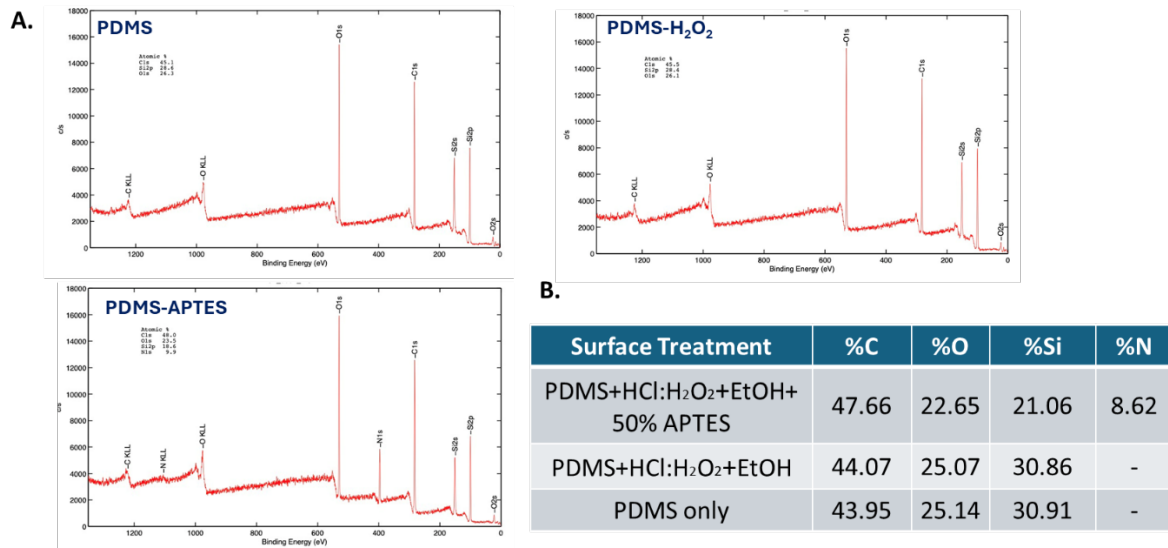


Figure 3-7: XPS analysis of surface modification. **A.** Curves showing the presence of certain atoms on the analysed surface and the binding energy of these atoms. **B.** Table showing the surface elemental composition of the PDMS.

The XPS curves were obtained for three PDMS films, with (i) no treatment, (ii) with surface activation by the H₂O₂:HCl:H₂O (1:1:1), and (iii) with surface modification by 50% APTES (**Figure 3-7.B**). It can be seen that the first two cases have almost similar surface composition indicating that the surface activation does not take part in modifying the surface. The increase in the carbon content in case (ii) is mostly due to the ethanol used for enhancing the surface hydrophilicity and sterilization of the chips. For case (iii) presence of bonded nitrogen (N) can be seen which comes mostly from the bound amine groups (-NH₂) on the surface from APTES. The percentage of this group is lower than expected mostly due to the time the experiment was performed is more than 24 hours from the surface treatment. This indicates that 8% Nitrogen present on the surface is part of a stable modification.

2.3 Bioink testing on the hydrogel testing chip

2.3.1 Live-dead viability analysis of encapsulated cells in bioinks

L929 cells mixed with the different bioinks in 10 million/mL were added to the surface-modified well bottom of the hydrogel testing chips. A 5 μ L of the hydrogel-cell mixture was added to the stepped well and crosslinked at 0.5 A, 1 A, or 1.5A input current. For control, cells were kept under the same conditions of light exposure without the hydrogel. A new bioink version D' was tested, which just had a double collagenase composition compared to its counterpart (bioink D). On day 1 after crosslinking, the cell viability was measured for all samples.

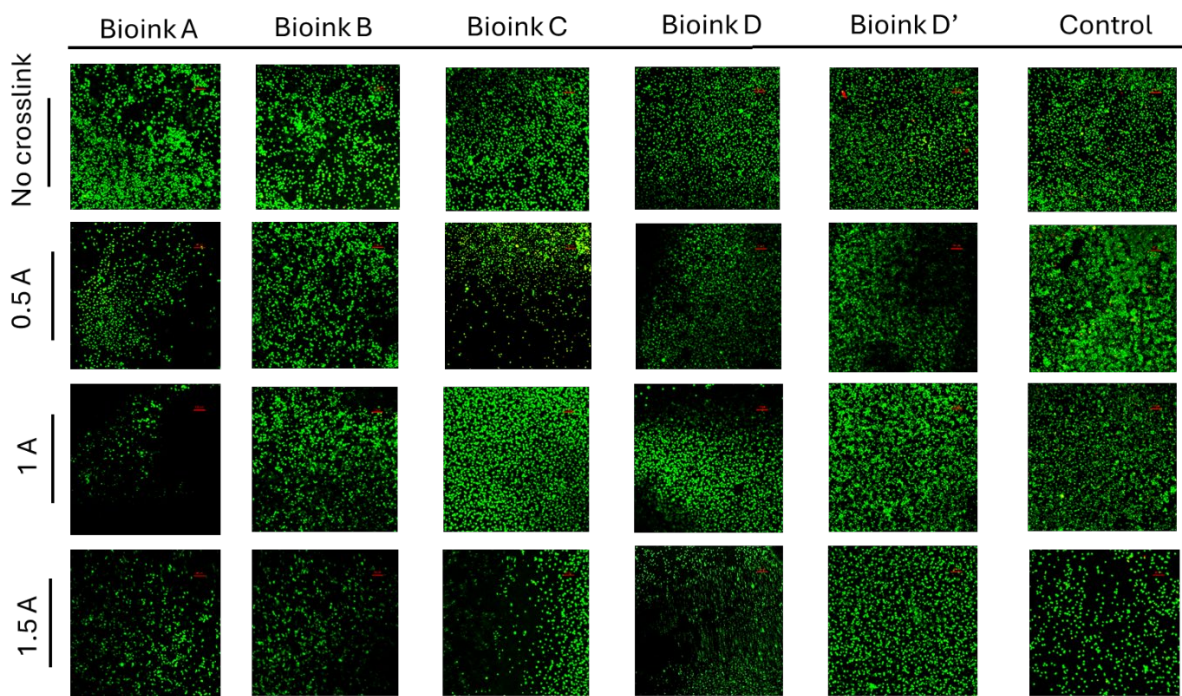


Figure 3-8: Live-dead test for cell viability of cells encapsulated in bioinks and crosslinked using different input current conditions of the CML.

For the Bioink A, the crosslinked hydrogel became too light and detached from the surface and floated in the medium, making image acquisition very difficult for the crosslinked cases (**Figure 3-8**). A similar problem was seen for more crosslinked versions of the bioinks (crosslinked at 1.5A) for bioinks B, C, and D. However, bioink D' seemed the best out of the other bioinks in terms of even spreading on the well surface and showed viable cells in encapsulation. The bioink D' formed a clear gel after crosslinking and was much less viscous compared to bioinks A and B (pepsin-digested bioinks). The floating bioinks indicated that the amine groups on the surface were not adequate for attaching the crosslinked bioink to the surface. Some methods need to be developed to enhance the APTES treatment efficiency and durability.

2.3.2 Viability analysis of encapsulated cells with CCK-8 assay

As described previously, CCK-8 could give a better understanding of the viability of the total cells in encapsulation, which may not be visible in the live-dead fluorescence assay due to differences in the planar positions of floating cells in the well. The test was performed in the hydrogel testing chips, but was transferred to a 96-well plate for taking the spectrophotometer reading. The cells not in any hydrogel and not exposed to UV were considered as controls.

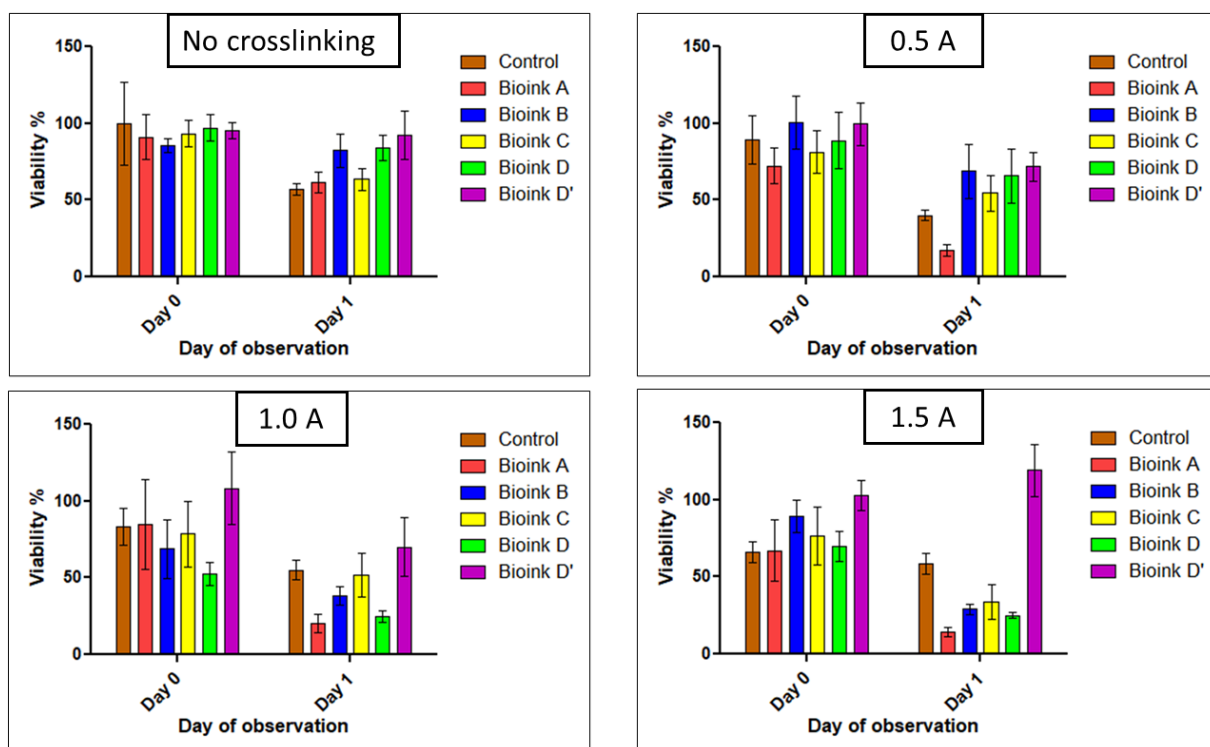


Figure 3-9: Cell viability and proliferation analysis of the hydrogels encapsulated in different bioinks crosslinked using different input currents of the CML using CCK-8 assay.

The results from the CCK-8 correlated with the live-dead viability test observations. Bioink D' was confirmed to be the most compatible bioink. For the partially crosslinked conditions (0.5A and 1A), the viability of the cells deteriorated over time for all bioinks, including bioink D' (**Figure 3-9**). However, for the well-crosslinked case at 1.5 A, cells in bioink D' found to be very good on day 0, and they even proliferated by day 1. These results indicated that bioink D' could possibly be used for droplet production tests.

2.4 Droplet formation for encapsulating cells in Bioink D'

Droplets were prepared using the OTF system with CML (at 1.5 A input current) and the long channel CC using bioink D'. Decently monodisperse droplets with even cell distribution could be produced with this bioink (**Figure 3-10.A**). However, the droplets did not show a stable shell even on Day 0. The higher flow rate of 50-100 (35 seconds exposure time) was worse in terms of crosslinking compared to the 25-50 flow rate with double the exposure time. On day 1, the droplets started dissolving (getting smaller) for the lower flow rate, with very poor cell viability. For the higher flow rate, they were almost completely dissolved. With so many components in the bioink, it was difficult to identify what was causing the inefficient crosslinking of the droplets.

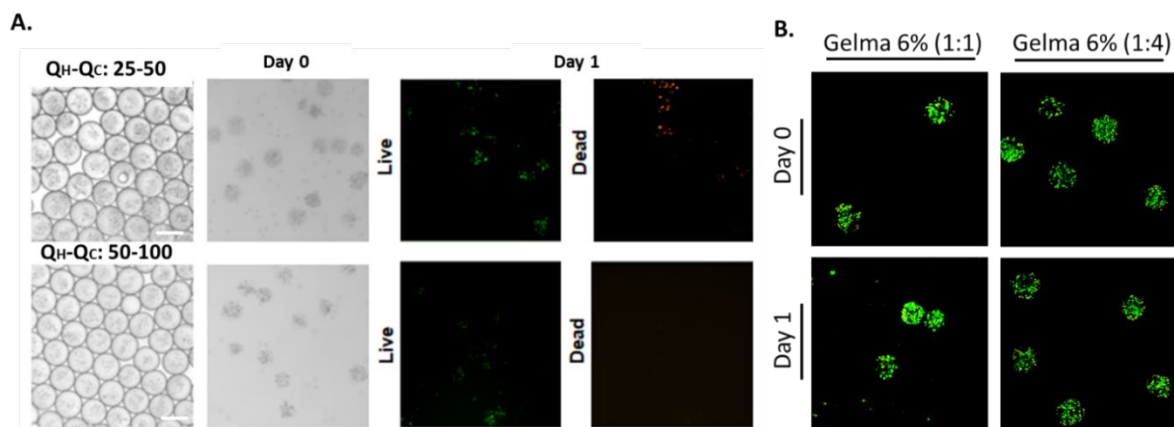


Figure 3-10: Droplet formation with bioinks and cell ratio optimization. **A.** Droplets produced from bioink D' at two flow rates with 177 mW/cm^2 (input current 1.5A) with droplets before and after washing on Day 0 and live-dead analysis on day 1. **B.** Live-dead and crosslinking analysis of GelMA droplets with two cell ratios in GelMA (ratio between a cell in media and to hydrogel).

The first tests were made to optimize the cell in media to hydrogel ratio and check if it affected crosslinking or cell viability of encapsulated cells or both. Initially, until this stage (for this chapter only), cells in media were mixed in a 1:1 ratio with the hydrogel. For these tests, only the GelMA part of the bioink was used at 6% initial concentration. To this 6% GelMA, cells in media were mixed in a 1:1 ratio or with 1 part of the cell in media to 4 parts of the hydrogel (GelMA). It was observed that droplets with a 1:4 ratio of cells in media to GelMA were much more stable on day 1 of culture. The 1:1 ratio droplets started dissolving and releasing cells to the external media in the process. Cell viability was also observably better in the 1:4 ratio droplets, but still, droplet death near the edges was observed. This ratio was henceforth used for all optimization studies (results from Chapters 1 and 2 as well). The reason for the improvements in viability and crosslinking was mostly because of the higher photoinitiator concentration due to less dilution compared to the 1:1 ratio condition.

2.5 Hydrogel optimizations for high-throughput droplet formation

2.5.1 GelMA and HAMA optimizations

Individual components were tested to obtain the best hydrogel composition for droplet formation and cell viability. Firstly, GelMA concentrations were optimized with 4%, 6%, and 8% of GelMA (initial) concentrations, resulting in final concentrations of 3.2%, 4.8%, and 6.4% GelMA. Among these 3 cases, the 6% GelMA samples showed the best viability with the formation of stable droplets using the CML at a higher irradiance of 218 mW/cm^2 (resistance of the lamp was altered to obtain higher irradiances during this stage) with an exposure time of 35 seconds (**Figure 3-11.A**). In the case of the 4% GelMA samples, the droplets formed were soft and easily degradable, leading to the release of cells to the surroundings within short culture periods (1-2 days). GelMA 8% however, appeared to be a bit too stiff and slowed down cell proliferation. Also, 8% GelMA had a higher viscosity and made droplet preparation difficult. For all cases, however, the “shell” with partially crosslinked GelMA was visible on Day 0.

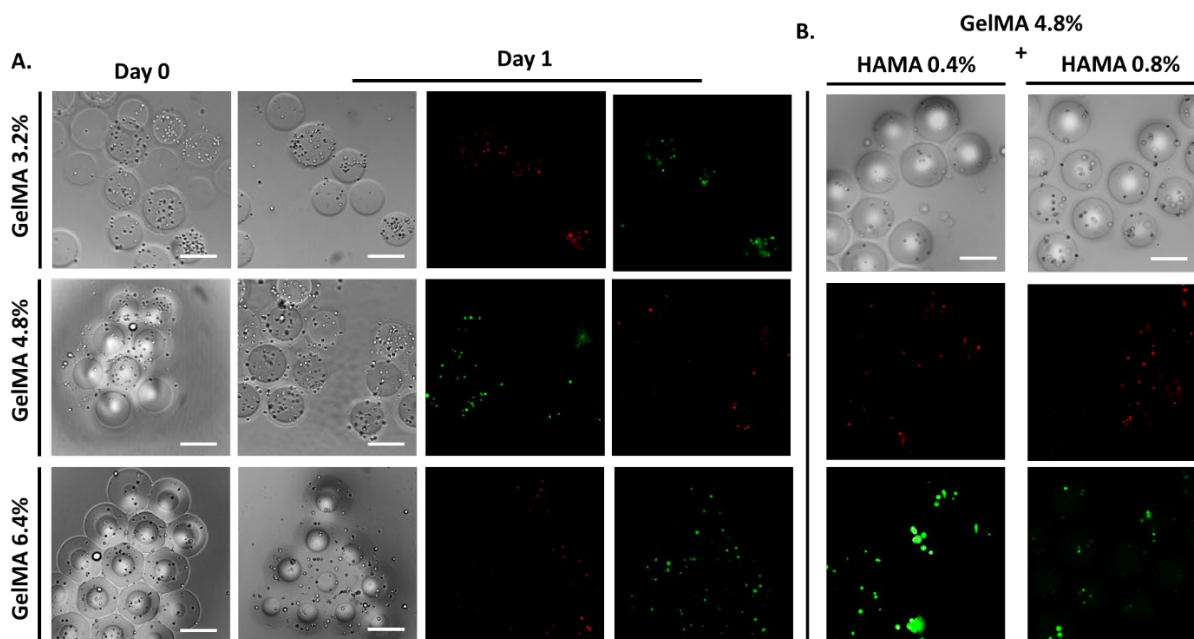


Figure 3-11: Optimization of GelMA and HAMA composition. **A.** Droplets produced with different GelMA concentrations on Day 0 (left) and their live-dead analysis on Day 1. **B.** Live-dead analysis of cells encapsulated in GelMA with different HAMA concentration droplets.

After finding the 4.8% GelMA to be the best GelMA concentration for better cell viability, the HAMA concentrations were tested with 4.8% GelMA. Also, in GelMA-HAMA droplets, the shell was visible after crosslinking (**Figure 3-11.B**). Among the two HAMA concentrations, 0.4% was found to be better compared to 0.8% (initially 0.5% and 1% HAMA), both in terms of viability and cell proliferation. Cell proliferation rate was found to be lower than only GelMA droplets, probably due to the interspaced HAMA linkages resulting in reduced pore sizes (but increased number of pores for cells to attach in).

2.5.2 Optimizations for better droplet formation with good cell viability

The next set of hydrogel optimizations was done after the CL optimizations and complete development of the OTF crosslinking system. The lower channel length CC with an exposure time of 15 seconds at flow rates Q_H - Q_O of 25-125 $\mu\text{L}/\text{min}$ was used at 884 mW/cm^2 of the CL₃₈₅ for the final optimization of hydrogel composition. The encapsulated cells were tested by live-dead viability test (quantified by manual measurement of fluorescent cells of each type) and correlated with CCK-8 assay results. The droplets formed had negligible “shells” at day 0 and maintained a stable structure for up to a week. However, since fast-growing cells (such as L929 fibroblasts or cancer cells) were used for encapsulation at this stage after day 5 cells start degrading the hydrogel matrix and migrating outside on and after day 5. Hence, viability in encapsulation was measured till Day 5 for these experiments. The breast cancer cell line, MDA-MB-231 cells, growing in the three different hydrogels have been shown below.

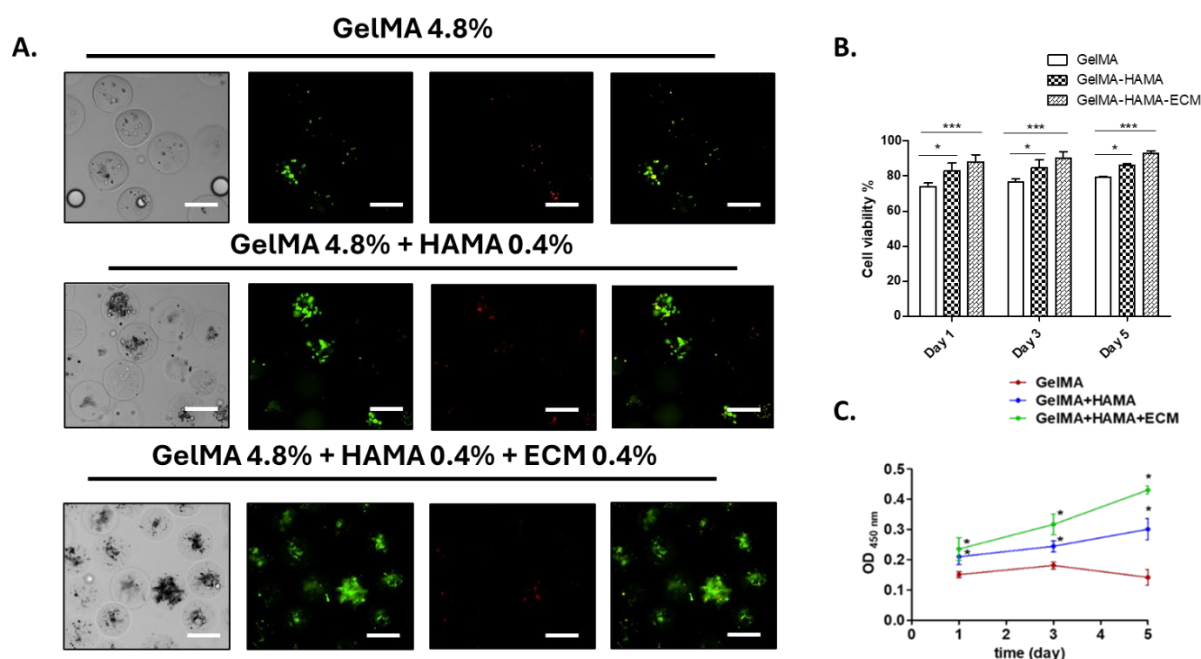


Figure 3-12: Hydrogel concentration optimization based on cell viability and proliferation. **A.** Live-dead analysis of MDA-MB-231 cells encapsulation in three different hydrogel compositions (Scale bar = 200 μ m). **B.** Quantification of viability based on fluorescent signal calculations. **C.** CCK-8 analysis of the encapsulated cells for days 1, 3, and 5.

Out of the three hydrogels, GelMA-HAMA-ECM was found to be the best for cell growth, followed by GelMA-HAMA and lastly GelMA. However, for all three cases, the average viability was above 75% (**Figure 3-12.B**). Proliferation rates were slowest in GelMA-HAMA hydrogels. However, cell proliferation was quite good in the ECM-containing hydrogel mix, mostly due to the growth factors from the dECM. Based on CCK-8 results, GelMA hydrogel showed a decline in growth by Day 5 (**Figure 3-12.C**) due to the migration of cells outside the droplet, which were not included in the assay (also not visible in **Figure 3-12.A** since droplets were sampled out of the culture dish and observed on a glass slide).

3 Discussion

[GelMA-based hydrogels for cell encapsulation in droplets]

GelMA-based hydrogels have been widely used for cell encapsulation applications due to their high stability, good mechanical strength, and ECM-mimicking characteristics. GelMA droplets with NIH/3T3 fibroblasts were prepared microfluidically and subsequently photocrosslinked on-chip in a couple of researches^{70,106}. In another study, coacervate microparticles were synthesized with a mixture of GelMA and oxidized methacylated alginate (OMA) for stem cell encapsulation and differentiation¹⁰⁷. The single-channel generator limited the throughput of the system. PEG and GelMA microspheres were demonstrated for encapsulation of several types of cells based on visible light photo-crosslinking¹⁰⁸. A non-microfluidic system was used for microdroplet generation with L929 fibroblasts in GelMA emulsified in soybean oil and photocrosslinked in UV (very similar to our stationary

crosslinking system)¹⁰⁹. However, the droplets thus generated were highly polydisperse. Other than droplets, GelMA has been used on micropatterned substrates, used for micromoulding, bioprinting, fiber formation, microfluidic systems (organ/vessel-on-chip systems) for growing a wide variety of cells such as fibroblasts, stem cells, and endothelial cells in encapsulation¹¹⁰.

[Importance of hydrogel screening for combinatorial hydrogels]

The purpose of having combinatorial hydrogels is to have the best properties of each component integrated together to have a superior mixture with the required characteristics. Initial inspection of the bioinks provided to us revealed that collagenase digestion, in addition to pepsin, produced more homogenous bioinks with reduced chunks/fibres of undigested dECM. A probable reason for this is the complete breakdown of the collagen triple helices from the ECM by collagenase, which remained preserved after pepsin digestion¹¹¹. Screening of hydrogels helped us analyse the basic physical and chemical properties of the hydrogels, such as viscosity, density before and after crosslinking, crosslinking times, and even viability of encapsulated cells. These factors not only helped us design the system parameters but also helped us find the most suitable hydrogel for cell growth and proliferation, which varies depending on the cell type. This makes the optimization process much simpler and time-efficient.

[PDMS chip hydrophilicity methods reported and necessity]

The PDMS chips that we developed for hydrogel screening could be rapidly prototyped, but suffered from the inherent hydrophobicity of the material¹¹². Hydrophilicity of the surface was necessary to allow even spreading of the hydrogel pre-polymer solution. Hydroxyl (-OH) groups could be introduced on the PDMS surface by O₂-plasma treatments, but the effect is not very durable (can be enhanced by keeping the surface dipped in water) and also makes the material less permeable to oxygen¹¹³. Additionally, the plasma discharge could not reach the well-surfaces in our design. However, the low durability of surface treatments was found in several of the other treatments attempted due to the non-covalent nature of the treatments, where the random Si-OH bonds on the surface get replaced by the oligomers from the PDMS bulk by rearrangements, resulting in the return of its hydrophobic tendencies. The biological surface treatments were tried mostly with nature-derived hydrogels but were found to unevenly modify the surface, possibly due to this hydrophobic recovery¹¹⁴. The structural inconsistencies and inadequate mechanical strength in the hydrogel to strongly adhering to the surface groups and needing thicker coatings, which was not possible to accommodate in our design¹¹⁴. For silanization-based modifications, the initial -OH groups on the surface are necessary for the covalent Si-O-Si bonds between the PDMS and the silane¹¹⁵. However, in our case, the first wet chemical activation with the H₂O₂: HCl: H₂O may not have been efficiently performed, leading to low efficiency of the APTES modification.

[GelMA in combination with HAMA for cell encapsulation]

The hydrogels that we screened were to have a hydrogel composition suitable for our final artificial organ development. The GelMA-HAMA combination was used to form interpenetrating networks of two hydrogels to allow a greater number of pores for cell adhesion and to increase the mechanical strength of the generated droplets. GelMA-HAMA droplets with chondrocyte spheroids were created by the hanging drop method, showing highly viable cultures with better physiological functioning by Wang and colleagues (2021)¹¹⁶.

Chondrocytes were also encapsulated in 3D-bioprinted GelMA-HAMA scaffolds with cellulose nanocrystals¹¹⁷. GelMA/HAMA bioinks with PEGDA crosslinker were also used for adipose tissue-derived stem cell culture¹¹⁸. Some drug-loading applications have been tried in GelMA/HAMA microspheres with conjugates/composites or in scaffolds for regenerative medicine and drug delivery^{119,120}. Testing of such scaffolds *in vivo* has shown great promise without triggering an immunogenic response.

[Organ-specific dECM for tissue engineering applications]

The applications of dECM in regenerative medicine have been wide due to its diverse composition of proteins such as collagen, elastin, laminin, fibronectin, and a wide variety of growth factors, receptors, and cytokines/chemokines¹⁰¹. The ECM performs several functions, such as providing a surface for cell adhesion, intercellular, biomechanical, and biochemical signalling, and providing enzymes for tissue remodelling. Decellularization is performed to remove cellular and nuclear components that might trigger immunogenic responses while the essential physico-chemical properties and structural integrity are preserved in the prepared dECM-based materials¹²¹. Organ-derived ECM has specific molecules and associated factors that maintain the organ's niche. Several bioinks with GelMA and/or HAMA have been prepared to serve as bioinks. A GelMA-based bioink was prepared with cardiac dECM for bioprinting cardiac patches¹²². In another study, a doubly crosslinked thiolated gelatin and HA hydrogel was used in conjunction with porcine liver-derived dECM¹²³. A combination of HAMA with cartilage-derived dECM was found to demonstrate chondrogenic differentiation in BM-MSCs¹²⁴. Similarly, we decided on integrating pancreatic dECM for our artificial islet droplets.

[Future perspectives]

The screening techniques currently being used for reproducible results are still dependent on conventional well plates, which show reliable results for bulk hydrogel (around 100 μ L). For small volumes analogous to our droplets, the PDMS chip would be better, and the durability and efficiency of the surface modification need to be improved by altering the initial wet activation method or by trying some other surface modification technique. In the case of the hydrogels, further conjugation of other functional groups can be attempted to improve crosslinking efficiency or cell viability enhancement.

Chapter 4 : Re-encapsulation of Droplets in Droplets for Compartmentalised Co-culturing of Cells

In an organ, multiple cell types co-exist to serve various functions in maintaining the structural and functional characteristics of the organ. For such organization, there are features such as membranes and linings to segregate the cells. For mimicking the physiological system, such segregated structural forms are required, which can be obtained through droplet microfluidics for generating complex droplet structures.

Complex droplets using Microfluidics

Microfluidics has facilitated the development of various designs to enable complex structures in terms of both shape and composition. Some of the attempted complexities have been described in the table below (**Table 4-1**).

Table 4-1: Types of complex microparticles that can be generated using microfluidic techniques.

Complexity type	Particle name	Fabrication method	Application	Reference
Shape	Plug and disk	Microspheres confined and deformed in channels, crosslinked in situ by UV or thermal crosslinking	Mimicking anisotropic biological cells or colloids	[1] ¹²⁵
	Disk array	Arranging disc-shaped droplets in regular periodicity within a channel	Grating or microlens array fabrication	[2] ¹²⁶
	Truncated spheres/hemispheres	A monomer and an immiscible, non-polymerizable phase, through a third aqueous stream in an FFD	Polymeric droplet generation with liquid cores	[3] ¹²⁷
	Crescent shaped	Microparticles with Eudragit® (co-polymer of acrylic and methacrylic acid) core, where the PLA shell was collapsed by drying	Yeast cell culture demonstrated	[4] ¹²⁸
	Rods, ellipsoids, discs	Volume manipulations in an FFD device to exceed that of the largest sphere that can fit in the outlet channel	Multi-material and multi-shape particles can be generated in high throughput	[5] ¹²⁹
Structure	Core-shell	Double emulsion W/O/W or O/W/O droplet formation in microfluidic channels with an inner core and outer shell in microfluidic channels	Biomedical engineering, emulsified structures in textiles, food, and cosmetics	[6]

	Janus particles	Two-faced droplets generated by Y-junction FFD with amphiphilic molecules	Micromotors, bioimaging probes, and solid surfactants	[7] ¹³⁰
	Ternary particles	Tri-phasic polymeric particles generated by introducing two different fluids in the central flow channel of an FFD, generating three parallel co-flow streams in the jetting regime	Multi-functional particles for complex analysis	[8] ¹³¹
Composition/ Functionality	Particle encapsulation	Polymerization of external material in the long, winding channel for higher residence time	Biological assays	[9] ⁹
	Ferrofluidic droplets	Cross-flow microparticle encapsulation in ferrofluid droplet and subsequent magnetic field-driven sorting in co-flow	Biochemical assays	[10] ¹³²
	Quantum dot (QD) barcoding	Multi-inner phase co-flow channels to generate polymeric shells with multiple QD cores	Spectral coding for biomedical applications	[11] ¹³³
	Nanoparticle synthesizing droplets	Pinching-off of droplets from co-lateral flowing streams with alkoxide solution and hydrolysing mixture to form nanoparticles inside the droplets	Nanoparticle synthesis, microreactor system	[12] ¹³⁴

Core-shell droplets: principles, strategies used and applications

The first double emulsion droplet production was discussed in a research by William Seifriz in 1925, where he demonstrated emulsification of ~1 mm oil globules in water while the oil globules contained a fine water core¹³⁵. However, these structures suffered from instability and oil density-dependent variations were discussed in this research. The basic principle for such droplet formations was explained by Matsumoto and colleagues in 1976 based on a two-step mechanism: (i) Generation of W/O emulsion based on Bancroft rule (depending on solubility of the surfactant in either of the phases), and (ii) formation of double W/O/W emulsion by using the previously generated W/O emulsion as the oil phase for the emulsification process¹³⁶. This process has been commonly used for core-shell nanoparticle formations. To attain monodisperse structures with even distribution of components, microfluidics has been the best solution.

Microfluidics allows a one-step generation of core-shell droplets with high reproducibility and throughput. The most commonly used microfluidic strategies to obtain core-shell droplets include¹³⁷:

- **3D-Flow focusing (3D-FFDs):** This is the most common design used for core-shell droplet formations. Immiscible fluids are injected into separate central and side channels of a microchannel. As the fluids converge and the section narrows, the flow becomes unstable due to Rayleigh–Plateau instability, creating uniform droplets at the focusing section. These droplets form double-emulsions as they disperse into the continuous phase. The parallel-flow and reverse-flow configurations are used depending on the outer and middle fluid flow directions.
- **Double junction channels:** Droplets in such geometries are generated by shearing the dispersed phase into the continuous phase. Droplet structure depends on interfacial tension and shear forces. Common designs include double-T-junction and double-cross-shaped microchannels. The devices have two junctions: one for oil droplet formation, and another for double-emulsion droplets. A double-FFD-based device with surface-modified conical capillaries coaxially embedded in square capillaries was also used for core-shell fabrication. Droplets form via drag and interfacial forces, with high inertia deforming and breaking droplets at specific points, allowing the other immiscible phase droplets to be injected into them.
- **Liquid- “chopping”:** Combining microfluidics with liquid-chopping technology, droplets are fabricated in three steps using a T-junction with a moving-wall structure. The dispersed phase is hydrodynamically focused and chopped to form inner droplets, which are injected into a continuous phase and further cut into double-emulsion droplets. The moving-wall mechanism is driven by pneumatic pressure, allowing precise droplet control.
- **Air-bubble driven:** This method uses compressed air bubbles to trigger droplet formation. In a T-junction-like setup, the dispersed phase forms a coaxial jet surrounded by the continuous phase. Air bubbles are introduced to block the continuous phase, and the fluid between the bubbles forms droplets. This process increases droplet formation frequency by adjusting the air flow rate.

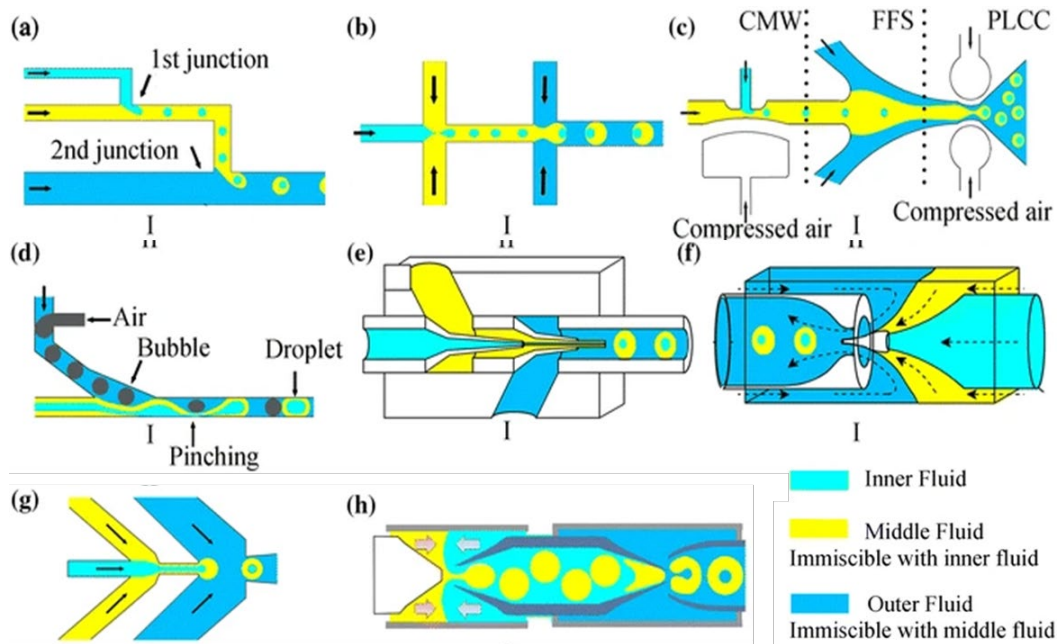


Figure 4-1: Mechanisms of core-shell droplet formation using different microfluidic channel geometries.

Such double emulsion core-shell particles have been used in a wide range of applications such as in drug delivery for purposes such as controlled drug release¹³⁸. These structures have been used in food sciences or cosmetics to prevent lipid oxidation¹³⁹. An interesting application of such droplets is seen as artificial cells with possibilities of developing a lipid bilayer at the W/O interfaces to mimic biological cells¹⁴⁰.

In our system, we tried to generate core-shell particles without using the oil phase as is used in W/O/W droplets traditionally, to avoid possible detrimental effects of the oil layer in the long-term cell cultures. Instead we converted the droplet formation into a two-step process similar to the process described by Matsumoto and colleagues as described above: firstly, the cores were produced in oil, crosslinked and washed to remove the oil; in the second step, the cores were dispersed in the second pre-gel layer made into core-shell droplets in oil and washed again to get a final oil-free system.

Cell segregation in tissue engineering

In the human body, tissues are made up of cell types required for specific functions. Instead of a random mix of cells, particular cell types are found within distinct boundaries in a specific arrangement, which is necessary for their function. Cell segregation is an essential feature in tissue engineering for serving the following purposes.

- **Promoting Functional Regeneration:** Spatial organisation of cells by controlling cell segregation is crucial for restoring function in damaged tissues or organs for regenerative medicine.
- **Cell-Cell Interactions:** Cell-cell interactions, such as adhesion and signalling, play a critical role in tissue formation and maintenance, for which segregation is required so as to avoid blocking of such interactions leading to reduction in functionality and/or assembly of the cells for tissue formation¹⁴¹.
- **3D Tissue Formation:** Cell segregation is particularly important in 3D tissue engineering, where the spatial arrangement of cells is critical for tissue function¹⁴². Complex 3D structures that mimic native tissues can only be created by controlling the cell segregation within the engineered structures.

The cell segregation in native tissues is regulated by molecules such as cadherins, which play an important part in signalling between cells for adhesion and segregation¹⁴³. For mixed cell populations without proper segregation, activation of Eph receptors and ephrins can result in cleavage of E-cadherin, causing repulsion between cells¹⁴⁴. The migration of cells based on these signalling molecules is driven by the regulation of the actin and myosin contractions triggered in the cytoskeleton. These contractions can also mediate boundary formation between different cell types. In our system, the core-shell geometry attempts to segregate the cells based on functionality to trigger better 3D tissue formation.

The droplets-in-droplet system was developed in this chapter, and some systems were demonstrated as applications of this system. A single T-junction with a slant leading to a widening channel was used to re-encapsulate the previously generated microdroplets (“cores”). The flow rates and droplet-to-shell pre-polymer ratio were optimized to allow efficient re-encapsulation. It has been demonstrated how each of the “shells” can encapsulate one or more “cores” and can even combine different “core” types within the same “shell”. GelMA was conjugated to fluorophores for these experiments. This droplets-in-droplet system was further used for culturing encapsulated cells, firstly with the model cells (L929 fibroblasts) in both the

core and shell, and cultured for up to two weeks. Complex co-culture model was attempted in this droplets-in-droplet system with different cell types in the cores and shell, respectively.

1 Materials and Methods

1.1 Re-encapsulation chip fabrication

The chip for the re-encapsulation of the droplets was fabricated similarly to the other methods described earlier in Chapters 1 and 2. Briefly, a PMMA sheet was micromilled to generate the master chip. A negative of this chip was created using PDMS and silanized for 4 hours at 10 mbar pressure to prevent the attachment of the PDMS copies from being generated. After silanization, the PDMS pre-polymer was poured into the area with the master chip's design and baked in a drying rack at 75°C for 4 hours. The chip was then removed from the negative mould, and inlets and outlets were punched using a 1 mm biopsy puncher. The chip and a glass slide were washed with soap, dried thoroughly, and then bonded using air plasma at <0.3 mbar pressure for 45 seconds. The water molecules generated as a by-product of this process were dried by heating the bonded chip over a hot plate at 120°C for 1 hour. The chip was cooled and then treated with Novec 1720 to make the channels fluorophilic, and the oil was dried at 75°C for 15 minutes.

1.2 Fluorescently-conjugated GelMA synthesis

GelMA was synthesized according to the protocol described in Chapter 3. Briefly, gelatin was dissolved in CB buffer and reacted with 0.01 mL of methacrylic anhydride/g of gelatin for 1 hour. The solution was dialysed for 3-5 days and freeze-dried to produce crosslinkable GelMA. For fluorescent conjugation of GelMA with Calcein (green fluorescence) and rhodamine (red fluorescence), the protocol described by Dong *et al.* and Qui *et al.* was used after slight modification^{145,146}. Briefly, 100 mg of calcein AM or rhodamine B was dissolved in 10 mL of dimethyl formamide (DMF) and reacted with 0.2 g of EDC (1-(3-(Dimethylamino)propyl)-3-ethylcarbodiimide) and 0.12 g of NHS (*N*-hydroxysuccinimide) for 4 hours (by continuous stirring) at room temperature to form the activation solution. Separately, 1 g of GelMA was dissolved in 10 mL of deionised water. To the GelMA solution, 1 mL of the activation solution was added and reacted overnight (12 hours) at 40°C. After completion of the reaction, the solution was dialysed again for 3-5 days (or until the unreacted dye was completely removed) and then freeze-dried and stored at -20°C.

1.3 Droplets-in-droplet production

The droplets-in-droplets production (also termed as re-encapsulation in this thesis) was done in the modified T-junction chip (fabrication process described in the previous section). The droplets generated by the OTF or stationary crosslinking were collected and thoroughly washed according to the washing protocol described earlier in Chapter 2 (using perfluorooctanol, Novec, and PBS sequentially). The final droplets in PBS were mixed with a hydrogel pre-polymer solution containing cells in the required concentration and loaded in the tubing, similar to the encapsulation method, and connected to the inlet for the hydrogel phase.

The syringe with Novec 7500 and 0.2% PFPE-PEG-PFPE surfactant was connected to the continuous phase inlet. As the hydrogel phase with droplets passed through the T-junction into the slanted and then the widened channels, the crosslinked droplets (“cores”) got encapsulated in the outer hydrogel droplets (“shells”) (**Figure 4-2.B.**). The droplets-in-droplets were collected and crosslinked by the stationary crosslinking method and washed similarly to the “core” droplets to remove oil and surfactant. After washing, they were transferred to cell culture media and placed in the incubator at 37°C with 5% CO₂ at 95% humidity.

1.4 Cell tracker staining and cell preparation for experiments

For distinguishing the different cell populations in the cores and shells, cell tracking dyes were used for staining cells. A 10 mM stock solution was prepared in DMSO for each of the red (CMTPX) and green (CMFDA) dyes (Thermofisher GmbH, Germany). For CMFDA, a 1:500 diluted solution (working solution) was made and added to the cells for staining for 45 minutes at 37°C. In case of the CMTPX dye, a working solution of the dye of 1:2000 (in media) was made from the stock solution. The staining protocol was the same as CMFDA staining. After incubation, the cells were removed from the culture dish by trypsinisation and used for experiments in the required concentration in the hydrogel pre-polymer solutions.

1.5 Image acquisition and analysis

The images with the fluorescent beads (and other images) were carefully obtained on the confocal microscope after adjusting the laser power and contrast settings on the Nikon A1 software. The obtained images were further processed and analysed using the ImageJ software.

2 Results and Inferences

2.1 Re-encapsulation system design and experiments

The chip for re-encapsulation was designed to facilitate encapsulation of the 200 µm droplets (“cores”). The inlet was slightly widened (500 µm) to prevent obstruction in flow due to clogging of the “cores” (**Figure 4-2.A.**). The rest of the T-junction part had a dimension of 200 µm × 200 µm. The final 0.16 sq. mm area of the channel had a slant to allow smooth breaking off into droplets and flowing of the “shell” droplet into the widened channel (1.2 mm) (**Figure 4-2.B.**). The number of cores that could be encapsulated in each shell varied depending on factors like flow rate and droplet packing fraction (discussed in section 2.2).

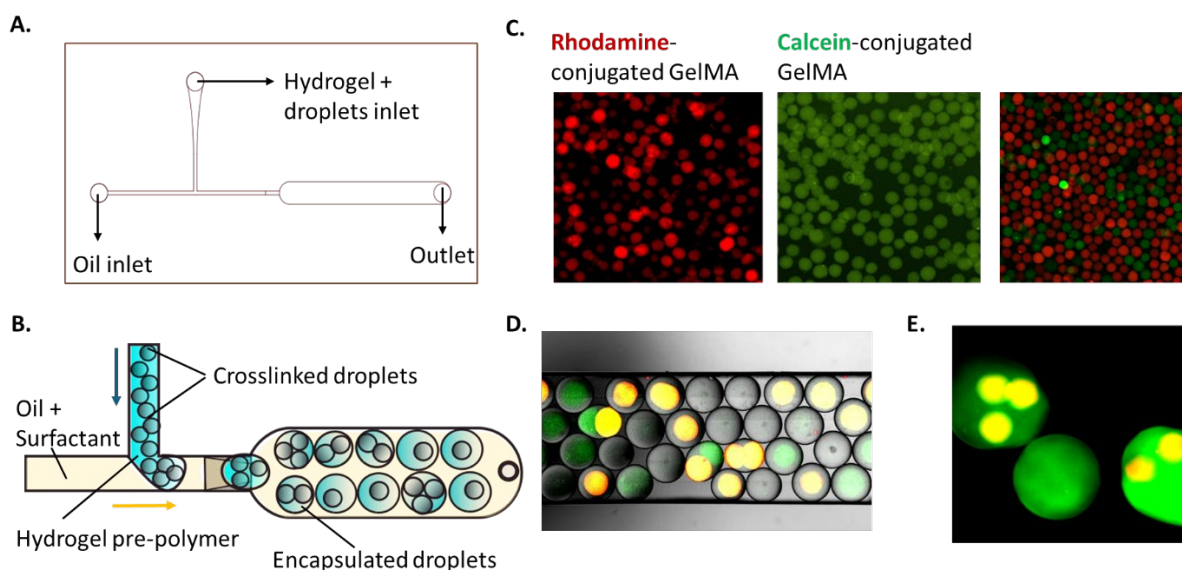


Figure 4-2: Re-encapsulation system design and analysis. **A.** Re-encapsulation chip design, **B.** Schematic of the re-encapsulation process, **C.** Fluorescently-conjugated GelMA droplets in PBS after crosslinking, **D.** On-chip view of the fluorescently-labelled GelMA droplets being encapsulated in colourless GelMA shells, **E.** Rhodamine-conjugated GelMA cores encapsulated in Calcein-conjugated GelMA shells.

For understanding the re-encapsulation process better, fluorescent conjugated GelMA was used to produce the “cores” (**Figure 4-2.C.**). The individual fluorescent “core” populations (of the same colour fluorescence) were tested to analyse the number of cores that could be encapsulated in a shell (similar to the system we were developing for the artificial islets) (**Figure 4-2.E.**). The other system was checked to find out if different core populations could be mixed and re-encapsulated in the shells. For this experiment, a mixed population of red and green fluorescent crosslinked cores was created and re-encapsulated. The view from the chip during the re-encapsulation process shows some of the droplets re-encapsulating both red and green droplets in the same shell (**Figure 4-2.D.**). The different core distributions have been shown in Section 2.3.

2.2 Effect of flow rate and volume ratio on droplet re-encapsulation ratio

The flow rates for re-encapsulation were selected based on a co-worker’s optimisations for the re-encapsulation of droplets. Flow rates of hydrogel with droplets (Q_{HD}) and of oil (Q_O) were either set to Q_{HD} - Q_O of 8 $\mu\text{L}/\text{min}$ - 8 $\mu\text{L}/\text{min}$ or 30 $\mu\text{L}/\text{min}$ - 120 $\mu\text{L}/\text{min}$. For the first experiments, L929 fibroblasts were used both for the core and the shell. The core droplets were made with 4.8% GelMA, while the shell was made of an initial concentration of 10% GelMA (finally 5% after mixing with the cores in PBS in a 1:1 ratio). It was observed that the slower flow rate (8-8) allowed formation of bigger droplets and showed a tendency of multiple droplet encapsulation (**Figure 5-2.A.**). The higher flow rate (30-120), on the other hand, mostly showed single or double core droplet encapsulation. Additionally, the packing fraction of the cores in PBS before mixing with the hydrogel pre-polymer solution had an effect on the shell size in general for both flow rates. This was logical since the lower the packing fraction of the cores, the higher the amount of PBS used to balance the volumes with the hydrogel pre-polymer for the shells, thereby diluting it. As a result, after crosslinking and washing, the droplets swelled more due to the larger pore size.

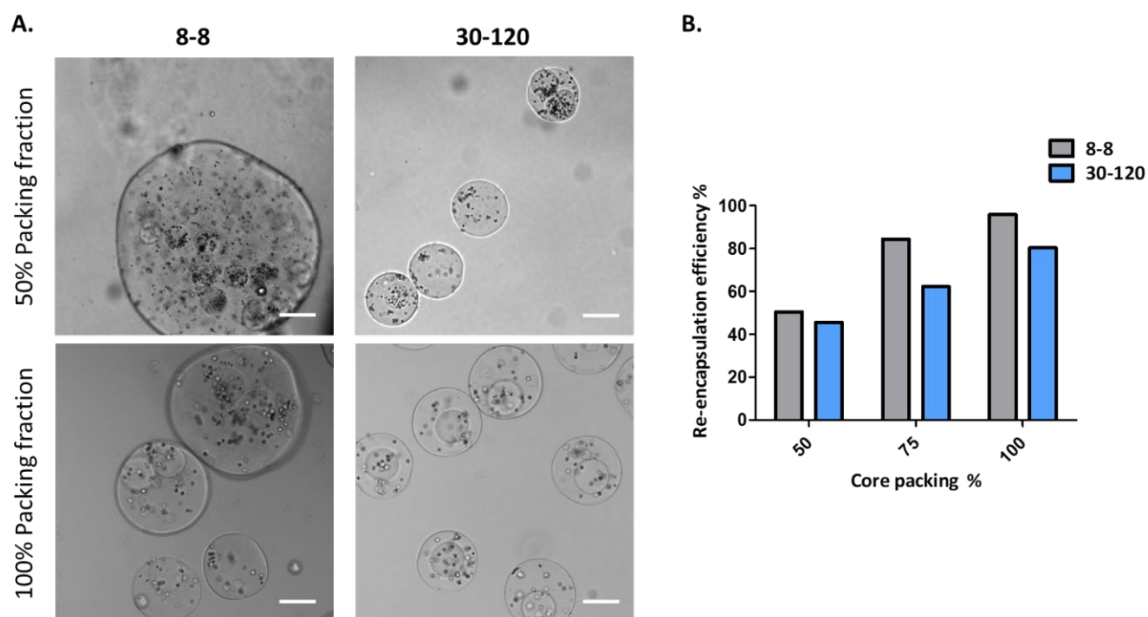


Figure 4-3: Packing fraction of cores and flow rates determine re-encapsulation efficiency. **A.** The visible difference between shell sizes due to variations in the core packing fraction after generation and washing on Day 0 (Scale bar = 200 μm). **B.** Re-encapsulation efficiency or the probability of a droplet (or more) getting re-encapsulated is determined by the core-packing percentage.

The packing fraction of the droplets determined the chances of one or more droplets being encapsulated in the shell. The number of shells with droplets compared to the total number of shells (empty shells + shells with encapsulated droplets) has been termed the re-encapsulation efficiency based on the following formula:

$$\text{Re-encapsulation efficiency \%} = \frac{\text{No. of shells with encapsulated cores}}{\text{Total no. of shells}} \times 100$$

The lesser the packing fraction (e.g., 50%: with 50% volume of beads + 50% volume of added PBS), the fewer the droplets available for encapsulation, hence leading to low re-encapsulation efficiencies (Figure 5-2.B.). The slower flow rates help in getting higher re-encapsulation efficiencies compared to the faster flow rates. This is due to the drag force acting on the slow-moving shells during formation, which pulls in cores inside it, thereby also increasing the probability of having multiple core encapsulation. However, the average shell size remains much more uniform for the faster flow rate, but also the chances of core droplets remaining as free droplets (without encapsulation) are higher in this case. Depending on the application, the flow rates can be decided on. In terms of re-encapsulation efficiency, however, the slower flow rate (8-8) at the highest possible core packing fraction (100%, only inter-droplet spaces filled with PBS) yielded a re-encapsulation efficiency as high as 95%.

2.3 Analysis of the re-encapsulation method: core distribution in different shells

As mentioned in the previous section, the slower flow rates (Q_{HD-QO} : 8-8 μL/min) could encapsulate higher proportions of multiple cores in a single shell. For the mixed population fluorescence-conjugated GelMA droplets encapsulation analysis, the slower flow rates were hence preferred. The 100% core packing fraction was utilised to ensure maximum re-encapsulation efficiency. It was observed that single droplet encapsulation was quite

common for both the fluorescent-conjugated droplets. In the case of double droplet encapsulation, both single fluorescent cores and mixed fluorescent core encapsulations were seen. Similar patterns of distribution were seen for triple droplet encapsulations, with more occurrences of mixed droplet populations in a single shell. From 4 or more droplets per shell, no more unfluorescent droplets were found in encapsulation.

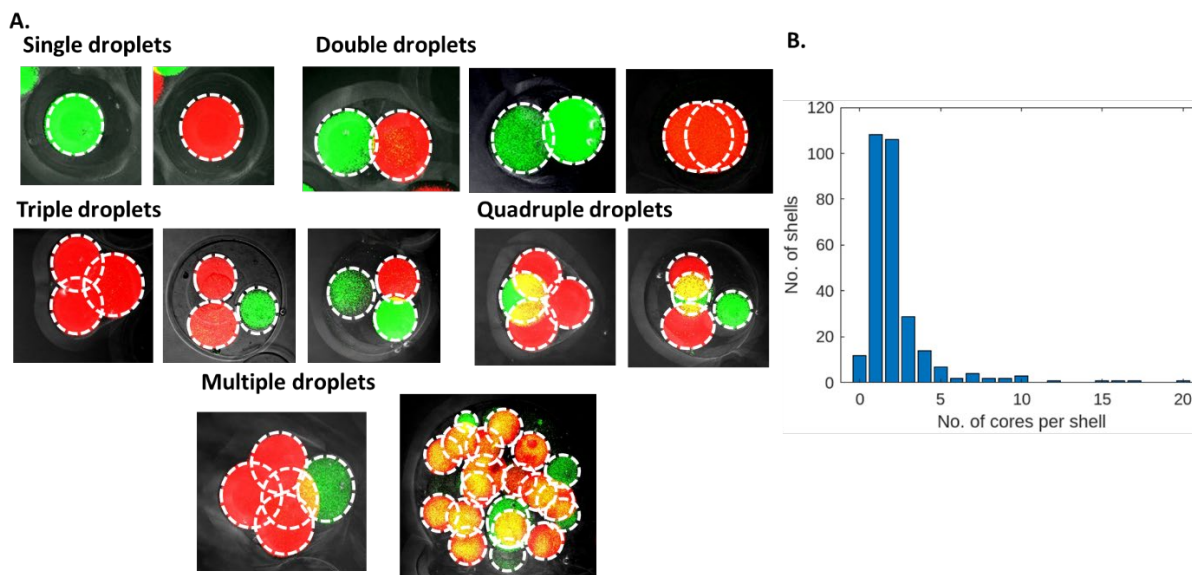


Figure 4-4: Core numbers and variations at maximum re-encapsulation efficiency. **A.** Encapsulation of cores of different fluorescence segregated in different numbers and orientations in different shells during the re-encapsulation process (core outline marked by white dashed line). **B.** Histogram showing the distribution of different numbers of cores per shell for the highest re-encapsulation efficiency process.

To analyse the frequency distribution of the number of cores encapsulated per shell, a histogram was plotted, calculated from 295 shells (**Figure 4-4.B.**). The occurrence of single or double droplet encapsulation was found to be the most common. Around 5% of the droplets had no cores encapsulated. A maximum of 20 droplets could be encapsulated in a single shell (picture shown in “Multiple droplets” (image on the right; **Figure 4-4.A.**).

2.4 Cell encapsulation in droplet-in-droplet systems

The initial tests for cell re-encapsulation were done using L929 fibroblasts as model cells, both for the core and shell. The cores were fabricated with 4.8% GelMA (initial concentration- 6%) while the shells were synthesized with 5% GelMA (initial concentration – 10%). The shells were crosslinked in a stationary state over the 385 nm lamp for 15 seconds. Not much variability in crosslinking was noticed during the shell crosslinking, probably due to their larger size and lower number (overlapping of droplets was fairly uncommon). The droplets were monitored for a period of one week in culture (**Figure 4-5**). The shell droplets maintained their structure throughout this period, indicating good crosslinking. The cells proliferated really well in both the core and shell. Many of the fibroblasts migrated out of the encapsulation and grew on the surface of the cores and shell, giving a tissue-like appearance to the structures. Live-dead assay performed showed the presence of mostly live cells.

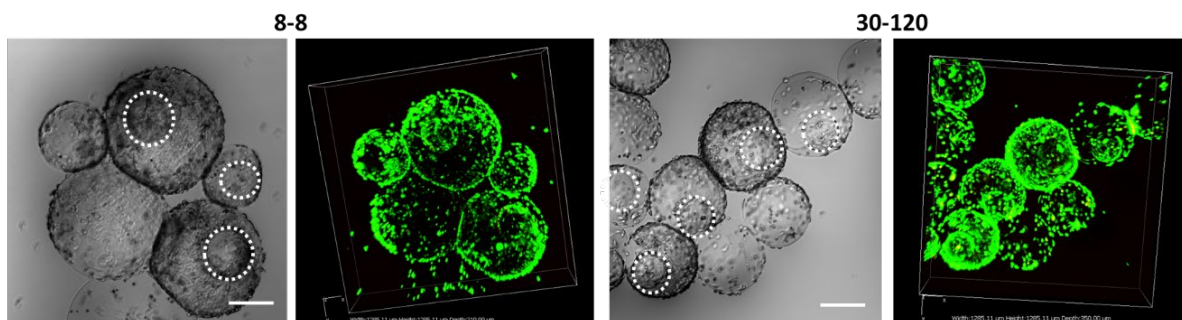


Figure 4-5: Cells cultured in re-encapsulation at the selected flow rates ($Q_{HD}-Q_0$). L929 fibroblasts grown in GelMA core and shell observed after live (green)-dead (red) staining on Day 7 of culture (Scale bar = 200 μ m).

2.5 Re-encapsulation attempts with different cell types in core and shell

The re-encapsulation system was tested with pancreatic islet β cells in the core encapsulated in shell with hDFs. For this test, the cores were fabricated with 4.8% GelMA with 0.4% each of HAMA and porcine pancreatic dECM. The RIN-m cells were labelled with CMTPX and encapsulated in the core at 10 million/mL concentration (The cell concentration was reduced for this test to check if overcrowding of cells in the core caused the escape of cells into the shell). For the shell, a cell pellet calculated at 10 million cells/mL of hDF was mixed with 10% GelMA and 1% HAMA (as in the previous section). The shell pre-polymer was then carefully mixed with the crosslinked cores generated by the OTF system in a 1:1 ratio and re-encapsulated on chip, followed by stationary crosslinking.

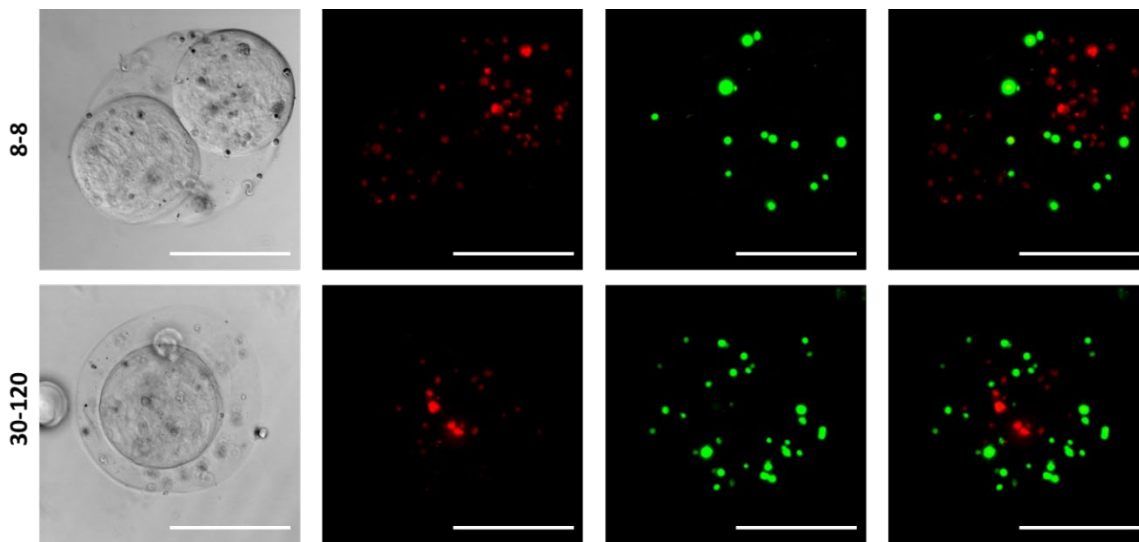


Figure 4-6: Re-encapsulation of pancreatic beta cells with hDFs. CMTPX-labelled RIN-m cells (red) in GelMA-HAMA-ECM cores re-encapsulated with CMFDA-labelled hDFs (green) in GelMA-HAMA shells on Day 3 of culture (Scale bar = 200 μ m).

The droplets were observed every alternate day to check for growth and proliferation (the cell tracking dye could be carried on for 7 generations). It was noticed on the very first day that a few of the red RIN-m cells were still present in the shell (**Figure 4-6**). This might be due to the several pipetting steps that are being performed or during the loading of the droplets into the syringe after mixing with the shell pre-polymer. Other than this issue, the re-encapsulation

was successfully carried out, and the core-shell droplets were cultured for more than two weeks with steady proliferation of the RIN-m cells and the hDFs, even though the rate of proliferation was reduced due to multiple composite hydrogel layers.

3 Discussion

[Core-shell droplets for cell encapsulation]

Double emulsion W/O/W core-shell droplets have been used previously for cell encapsulation applications, such as the liquid core-alginate shell microparticles developed by Nguyen and colleagues. The droplets were generated by electrospinning with coaxial nozzles. The PC12 cells cultured in the media core showed ~80% for 3 days in culture. In another liquid core system with an alginate-chitosan-alginate shell, encapsulated embryonic stem cells showed higher viability and reduced binding of immunoglobulin G (IgG) due to the selectively permeable shell¹⁴⁷. Co-culturing of HepG2 and 3T3 cells in a liquid core with alginate shell crosslinking mediated by HEPES¹⁴⁸. Such core-shell alginate structures have also been shown to facilitate vitreous cryopreservation of porcine adipose-derived stem cells, resulting in very low levels of cryoprotectant penetrating through the cell membrane, thereby reducing risks of cytotoxicity¹⁴⁹. In all the above-mentioned cases, only liquid cores have been used for culturing of the cells, while the hydrogel shell just acts as a boundary.

[Water-in-water-in-oil droplets]

A W/W/O structure was reported generated by a focused flow of the core aqueous phase and a coaxial flow of alginate and oil phase with Ca^{2+} ions for simultaneous crosslinking of the alginate while breaking into droplets¹⁵⁰. This system was used for embryonic carcinoma cells encapsulations, which showed embryoid body formation after the second day of culture. A similar system has been reported previously for generating an artificial liver in a drop, where different cells were grown in the cores and shells¹⁵¹. The hepatocytes in a liquid core and fibroblasts in the alginate shell system show enhanced albumin and urea metabolism compared to only hepatocyte cultures. Shifting from liquid cores, a collagen core (with or without alginate in it) and an alginate shell were used for culturing mouse embryonic stem cells¹⁵². A similar collagen-alginate core-shell system was used for in-droplet MSC differentiation for bone tissue regeneration¹⁵³.

[Factors affecting the core-shell structures: Necessity of oil removal]

The W/O/W double emulsions cannot facilitate long-term culturing of cells due to the presence of the intermediate oil layer. This layer prevents transport of the water-soluble nutrients or gases from the external media into the encapsulated cells¹⁵⁴. Even though some of the molecules can pass through this barrier by diffusion but the rates are insufficient¹⁵⁵. Additionally, most of these core-shell droplets use alginate shells, which are not suitable for all types of cells. For softer materials like GelMA, as in our case, the pore size is greater with higher biodegradability, and cells can migrate to the surface of the droplets. The cells can come into contact with the oil layer at this point if it is not removed and resulting in cytotoxicity.

[Core-shell particles for cell culture applications with a specific focus on this project]

Hydrogel core-shell microdroplets have been recently used for generating biomimetic disease models for cancer. GelMA hydrogel core-shell droplets were used for generating tumour models with A549 cells¹⁵⁶. Another study showed the development of a core composed of a mixture of collagen, alginate, and reconstituted basal membrane, while the shell was made of alginate to encapsulate MCF-7 cells for modelling breast cancer¹⁵⁷. Core-shell structures have also been used for β -cell encapsulation applications. A core-shell type microparticle generation technique was developed using a microfluidic electrospray device for encapsulating pancreatic β -cells¹⁵⁸. The core consisted of carboxymethyl cellulose with β -cells, while the shell was composed of alginate with cellulose nanocrystals providing structural durability and immunoprotection when implanted in mice. Double emulsion microdroplets with GelMA and PEGDA were produced for encapsulating pancreatic β -cells (INS-1 cells)¹⁵⁹. The microcarriers were implanted in mice and showed efficient glucose control at day 28 through the intraperitoneal glucose tolerance test.

[Possible improvements to the system]

The system currently involves a multi-step process to form the core-shell droplets. The system could be improved by connecting the core and shell formation steps into a single process. To enable this, the intermediate washing step for the cores needs to be simplified. The integrated system could increase the robustness of the process due to automation. Additionally, due to the avoidance of pipetting, droplet breaking will be reduced, and the presence of cells from destroyed cores can be prevented in the shells.

PART-B:

Application in

Cancer Models

and Artificial

Pancreatic Islets

Chapter 5 : Development of 3D Cancer Cell Models in Microdroplets

Among the most prevalent diseases in the world, affecting a significant part of the human population, cancer is of great significance. Cancer refers to a heterogeneous group of diseases marked by the dysregulated proliferation of abnormal cells that evade normal growth controls. These malignant cells can infiltrate surrounding tissues and, through processes such as metastasis (spreading), colonize distant organs, ultimately disrupting physiological function and posing a significant threat to survival. To investigate the progression of cancer and test treatment options, it is very important to develop disease models. Droplet microfluidics can serve as a tool to develop such models in a time-efficient manner.

Cancer: Subtypes and Prevalence

Cancer cells can develop in different organs in the body, and depending on this, they have been classified into different subtypes. The activity of these cells and the severity of the disease can depend on their location within the body. **Table 5-1** below summarises the organ-based subtypes of cancer, how they affect prognosis/mortality, and their prevalence globally and in specific populations.

Table 5-1: Cancer subtypes, the organs they affect, the prognosis of the disease, and the numbers and type of people they affects^{160–162}.

Cancer Type	Primary Organ Affected	Prognosis / Mortality Insights	Estimated Global Incidence (2018)	Typical Population Affected
Lung Cancer	Lungs	High mortality; most cases are detected at advanced stages with poor survival.	~2.09 million cases	Higher incidence in males; strongly linked to smoking and pollution.
Breast Cancer	Breast tissue	Good prognosis if detected early; poor survival if diagnosed late.	~2.09 million cases	Predominantly affects women; incidence is rising globally.
Prostate Cancer	Prostate gland	Often slow-growing, localized forms have excellent survival rates.	~1.28 million cases	Affects older men; more common in developed countries.
Colorectal Cancer	Colon and rectum	Moderate mortality; outcomes depend heavily on stage at diagnosis.	~1.10 million cases	Affects both sexes; risk increases with age and dietary habits.
Stomach Cancer	Stomach	Often diagnosed late; prognosis is poor in advanced stages.	~1.03 million cases	More common in men; high rates in East Asia and Eastern Europe.

Liver Cancer (HCC)	Liver	Aggressive course; late diagnosis is common, leading to low survival.	~0.84 million cases	Higher burden in Asia and sub-Saharan Africa, linked to hepatitis B or C.
Pancreatic Cancer	Pancreas	Very poor prognosis; rapid progression and low 5-year survival (~13%).	~0.39 million cases	Affects both sexes; incidence increases with age and family history.
Skin Cancer	Skin (melanoma and others)	Non-melanoma types are often curable; melanoma can be life-threatening.	~3.0 million non-melanoma; ~0.29 million melanoma	Higher incidence in light-skinned individuals; more common in older adults.
Non-Hodgkin Lymphoma	Lymphatic system	Prognosis varies by subtype; some aggressive, some indolent.	~0.51 million cases	Slight male predominance; affects both young and older populations.
Leukaemia	Blood and bone marrow	Outcomes depend on subtype; acute types progress rapidly.	~0.43 million cases	Occurs in all age groups; some types are more common in children.
Cervical Cancer	Cervix	Preventable; HPV vaccination and screening have reduced incidence in some areas.	~0.57 million cases	Affects women, particularly in regions with limited access to healthcare.
Thyroid Cancer	Thyroid gland	Generally favourable prognosis; often slow-growing.	~0.57 million cases	Affects women more frequently; common in younger adults.

Cancer cells vs Normal cells

As mentioned above, cancer is marked by uncontrolled proliferation of cells due to loss of the checkpoints to control growth and multiplication generally present in normal cells, and by avoiding programmed cell death or apoptosis, which defective cells exhibit in our body. Some of the other differences observed between normal and cancer cells are as follows:

- **Differentiation and Function:** While normal cells mature into specific, functional types, cancer cells often remain undifferentiated and immature, lacking the specialisation necessary for normal physiological roles¹⁶³.
- **Invasion, Metastasis and Adhesion:** Unlike normal cells, which adhere to their tissue of origin, cancer cells lose their adhesive properties and gain invasive characteristics to penetrate into surrounding tissues, and metastasize to distant organs¹⁶⁴.
- **Angiogenesis and Metabolic Adaptation:** Cancer cells continuously stimulate the formation of new blood vessels (angiogenesis) and shift their energy production toward

less efficient glycolysis (the Warburg effect), even under aerobic conditions—unlike the regulated metabolism of normal cells¹⁶⁵.

- **Morphological and Genomic Alterations:** Morphologically, cancer cells often display irregular shapes, enlarged/multiple nuclei, and disrupted nuclear architecture, reflecting their genomic instability. They accumulate mutations, chromosomal abnormalities, and epigenetic changes that further propel malignancy^{166,167}.
- **Immune System Interaction:** Normal cells are monitored and regulated by the immune system when necessary. Cancer cells, however, develop mechanisms to evade immune detection, such as altering surface proteins or secreting immunosuppressive factors¹⁶⁸.

Tumour Microenvironment (TME): Composition and role in progression

The tumour cells do not exist in singular populations but instead grow as a part of a complex ecosystem of non-cancerous cells, blood vessels, and extracellular matrix (ECM) that surrounds it, forming the tumour microenvironment (TME) (**Figure 5-1**). The TME plays a significant role in the growth, metastasis, and response to therapy in tumour cells¹⁶⁹. The concept of TME playing a role in cancer progression was proposed by Stephen Paget in 1889 through his “seed and soil” hypothesis, stating how interactions of the cancer cells (seed) with the host organ (soil) could promote metastasis¹⁷⁰.

The TME can be broadly classified into cellular and non-cellular components^{169,171}. The cellular components include cancer-associated fibroblasts (CAFs), cancer-associated adipocytes (CAAs), mesenchymal Stem Cells (MSCs), and immune cells such as tumour-associated macrophages (TAMs), neutrophils, myeloid-derived suppressor cells (MDSCs), regulatory T cells (T_{regs}), cytotoxic (CD8⁺) T lymphocytes (CTLs), dendritic cells (DCs), natural killer (NK) cells, and mast cells.

The non-cellular components of the TME include extracellular matrix (ECM), extracellular vesicles/exosomes, signalling molecules such as cytokines (e.g., IL-6, IL-8, TGF- β), growth factors (e.g., VEGF, PDGF, EGF), chemokines, and immune checkpoint ligands like PD-L1. The chemical/biochemical environment in which these cells are present also contributes to the TME.

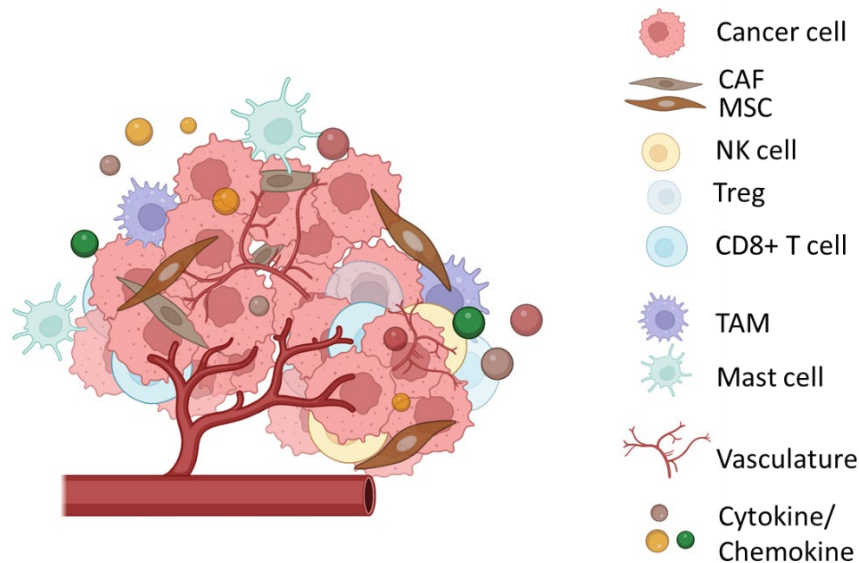


Figure 5-1: The different components of the tumour microenvironment (TME) (Created in <https://BioRender.com>).

The TME plays a very crucial role in the progression of cancer via the following mechanisms:

- **Angiogenesis and Hypoxia**

- As tumours grow beyond their oxygen supply, a state of hypoxia is created, triggering hypoxia-inducible factors (HIFs) that upregulate vascular endothelial growth factor (VEGF) and other pro-angiogenic factors, resulting in angiogenesis to promote tumour growth and metastasis¹⁷².
- Immune cells such as the TAMs and Mast Cells also amplify angiogenesis by secreting VEGF, FGF, MMPs, and other factors¹⁷³.

- **ECM Remodelling and Mechanotransduction**

- The ECM becomes stiffer due to collagen deposition and crosslinking (via the enzyme lysyl oxidase or LOX), creating physical paths that promote invasion and metastasis¹⁷⁴.
- Proteases like MMPs degrade ECM and release embedded growth factors, enhancing tumour spread¹⁷⁵.

- **Immune Suppression and Evasion**

- CAFs produce TGF- β and other cytokines that suppress T cell and NK cell function, recruit T_{regs}, and reduce CTL infiltration¹⁷⁶.
- TAMs secrete immunosuppressive cytokines (e.g., IL-10, TGF- β), express immune checkpoint ligands (PD-L1, B7-family), and drive immunotherapy resistance. Also, MDSCs inhibit antitumour immunity across multiple immune cell types^{177,178}.
- The ECM can physically block immune cell infiltration, and acidic conditions further hinder immune responses¹⁷⁹.

- **Metabolic Adaptations and the Biochemical Landscape**
- Low oxygen and acidity shift cancer cell metabolism, increasing glycolysis and lactate production, selecting for more aggressive phenotypes¹⁸⁰.
- Lactate accumulation can also polarise macrophages toward the tumour-promoting M2 phenotype¹⁶⁹.

Cancer Modelling: Requirements and Complexities

An effective disease model for cancer must replicate the fundamental hallmarks of tumour biology, including sustained proliferation, evasion of apoptosis, angiogenesis, invasion, and metastasis. To be clinically relevant, the model should also reproduce the tumour microenvironment, encompassing immune cells, stromal components, and extracellular matrix interactions. Genetic and epigenetic fidelity to human tumours is essential to ensure translational applicability, especially in preclinical drug screening and mechanistic studies.

However, developing such models is inherently complex due to the heterogeneity of cancer across and within tumour types. Capturing the dynamic interactions between cancer cells and their microenvironment, as well as interpatient variability, poses significant challenges. In vivo models, such as genetically engineered mice or patient-derived xenografts, require intricate design and long timelines, while in vitro systems often lack the full physiological context. Balancing model simplicity for experimental control with biological accuracy remains a central difficulty in cancer research.

In this chapter, we demonstrate how the high-throughput system and re-encapsulation techniques were used to encapsulate cancer cells with or without cells from their TME. It has also been shown how microfluidics helps solve the complexity of generating such models. These models were used to check for spheroid formation or drug testing in 3D culture systems, providing high physiological relevance where the hydrogel mimics the ECM.

1 Materials and Methods

1.1 Cell culture

The chronic myeloid leukaemia (CML) cells, K562, and their CAFs, HS-5, were obtained from Nencki Institute of Experimental Biology, Poland (*the project was done in collaboration with them). The cells were cultured in T75 flasks with Iscove's Modified Dulbecco Medium (IMDM). For the subculture of the K562 cells, which grow in suspension culture, a 2 mL volume of the cells with medium was transferred to 8 mL of fresh medium. The HS-5 cells were sub-cultured by trypsinisation. Sub-culture was done every 3-4 days.

The MDA-MB-231, HeLa-GFP and hDF-RFP cells were cultured in DMEM medium and sub-cultured similarly to the HS-5 cells.

1.2 Hydrogel preparation for experiments

Lyophilised GelMA was dissolved in 6% or 10% w/v in PBS, and lyophilised HAMA was added according to requirement (0.5% or 1%). The lyophilised powder was dissolved at 50°C for 1 hr, stirred at 200 rpm. The solution was filtered before experiments with a 0.22 µm syringe filter and stored at 37°C until the experiments. Cells in culture media were mixed with the hydrogel in a 1:4 ratio. For co-culture experiments in a single droplet, the cells were mixed in a 1:1 ratio before mixing with the hydrogel.

1.3 Generation of microdroplets for cancer modelling

Microdroplets were generated using the circular, step-emulsification chip at hydrogel and continuous phase flow rates of 50 and 150 µL/min. The droplets were collected in a 10 mm Petri dish and crosslinked using the stationary method for 10s using 884 mW/cm² of the 385 nm UV lamp. The droplets were washed sequentially with Novec-PFO, Novec, and PBS before transferring to cell culture media and culturing in the incubator at 37°C with 5% CO₂ with 80% humidity.

1.4 Cell interaction analysis and drug testing

For cell interaction analyses in the co-culture models, K562 and HS-5 cells were stained with the cell tracker dyes CMTPX (red) and CMFDA (green), respectively, and incubated for 30 minutes before the experiments. The fluorescence was observed at 560 nm and 488 nm, respectively, using the confocal microscope.

Once visible spheroids and cell interactions were observed in the droplets, the drug, Imatinib, was added in 0.5, 1, and 2 µM concentrations to the droplet samples. The cells were treated with the drug for 48 hours.

1.5 Cell viability and proliferation analysis

Cell viability in the samples was measured using the Live/Dead assay kit (Thermofisher GmbH, Germany). Live cells could be visualised at 488 nm (green fluorescence) while dead cells could be observed at 560 nm (red fluorescence) using the fluorescent or confocal microscope.

For proliferation, the CCK-8 assay was performed by adding 10 µL of the reagent to 90 µL of the droplets in the media. The absorbance of the samples was measured at 450 nm.

1.6 Complex models with droplets-in-droplet

To generate the complex models, the encapsulation was done as described in section 1.3. After washing with PBS and removing it, a 1:1 solution of the droplets was made with the hydrogel pre-polymer with a different cell type. The T-junction chip with the widened channel, as described in Chapter 4, was used with a hydrogel to a continuous phase flow rate of 30 to 120 µL/min. The droplets were again crosslinked by the stationary method at 884 mW/cm² irradiance over the 385 nm lamp for 10s.

2 Results and Inferences

2.1 Chronic myeloid leukaemia (CML) model for drug resistance test

2.1.1 Hydrogel optimization for cell encapsulation

Hydrogel screenings were done in bulk hydrogel blocks for cell encapsulation before every application. A systematic hydrogel screening was done for the experiments with the leukaemia cells (K562) and the bone marrow fibroblasts (HS-5) discussed in Chapter 1¹⁸¹. Different GelMA and GelMA-HAMA hydrogel compositions were tested for each of the cell types in 96-well plates. It was found that cell migration to the well bottom was more common in the fibroblasts compared to the K562 cells and happened faster in the lower hydrogel concentrations (**Figure 5-2.A**). Based on the cell viability and cell migration, 4.8% GelMA and 4.8% GelMA with 0.4% HAMA were found to be the best hydrogels.

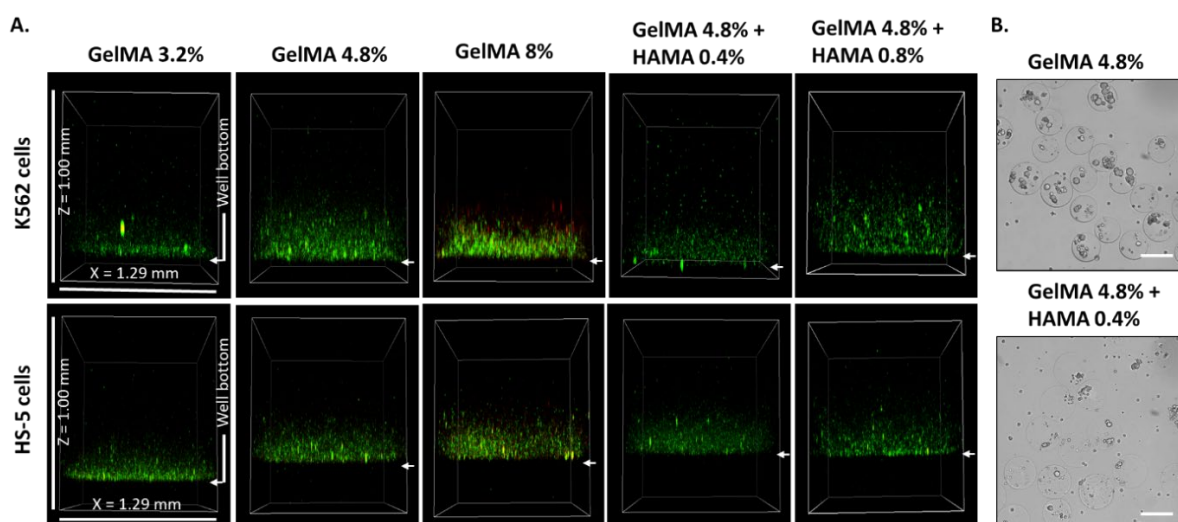


Figure 5-2: Hydrogel screening for leukaemia cells testing in 3D culture. **A.** Culture of K562 (leukaemia) and HS-5 (fibroblasts) cells in different hydrogels and tested for live (green fluorescence) and dead (red fluorescence) viability analysis on Day 2 of culture. **B.** Spheroid formation was seen in abundance in GelMA droplets than in GelMA-HAMA ones for K562 monocultures on Day 3 of culture.

On further investigation, at 8 million/mL cell concentration for K562 cells, droplets made from these two hydrogel compositions showed an observable difference in spheroid formation by Day 3 of culture (**Figure 5-2.B**). Hence, 4.8% GelMA was finally selected for further experiments to investigate the drug resistance in mono vs co-cultures (with HS-5).

2.1.2 Cell number optimization and spheroid formation

Initially, for experiments, a cell concentration of 12 million per mL was used for encapsulating the K562 cells in 4.8% GelMA droplets. However, due to the rapid proliferation of cancer cells and their membrane degradation mechanisms, it was observed that a large number of cells migrated out of the droplets as their population increased within the droplets (**Figure 5-3.A**). Hence, it was important to lower the cell number. However, on attempting low cell concentration (6 million cells per mL), it was observed that spheroid formation rates were lower and also slower than higher cell loading concentration (**Figure 5-3.B**). It was hence

estimated that 8-10 million cells per mL was ideal for generating the droplets to ensure good spheroid formation and lower cell migration rates till the drug test was performed.

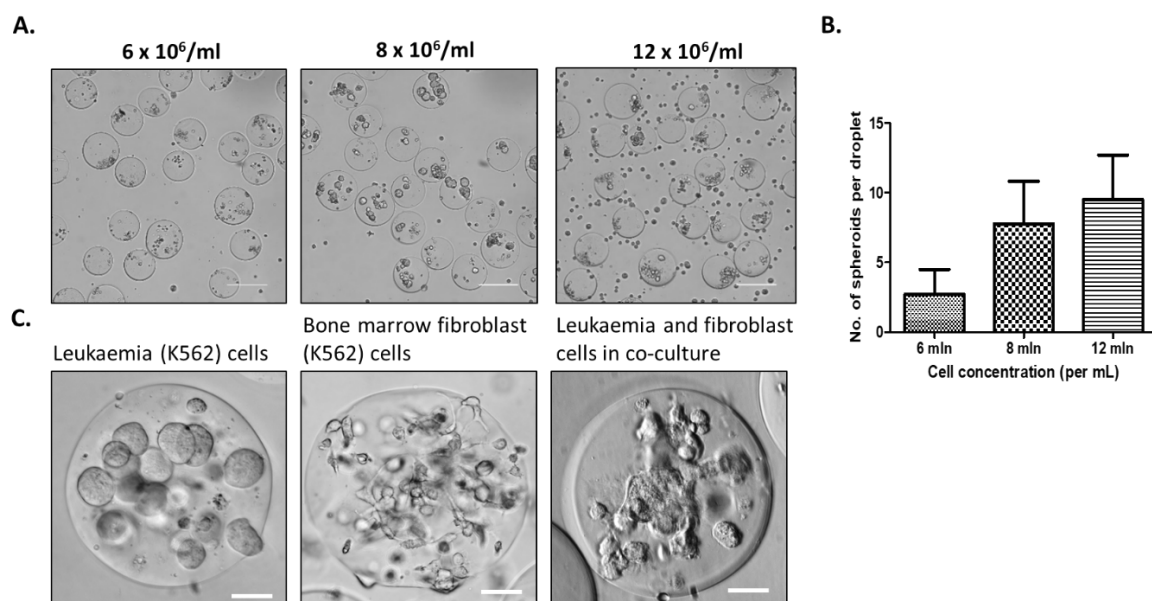


Figure 5-3: Spheroid formation by CML in microdroplets **C.** Spheroid formation in monocultures of leukaemia cells (K562) and bone-marrow fibroblasts (HS-5) and their co-cultures in GelMA 4.8% droplets (Scale bar = 25 μ m).

It was observed on day 2 of culture that these could form well-defined spheroids in GelMA 4.8% (synthesized with CB buffer protocol). However, the spheroid formation nature varied depending on the type of cell (**Figure 5-3.C**). While the leukaemia cells formed well-rounded circular spheroids, the fibroblasts showed more cell clustering than spheroid formation. The co-culture again showed a different form of spheroid, with the K562 spheroids being held together by fibroblastic tissues. The cells, however, start degrading the hydrogel and escaping from day 3 of the culture. This experiment showed that spheroid type also depends on cell type and not only on the hydrogel composition.

2.1.3 Cell interaction in co-culture models and drug resistance

For the co-culture model, the K562 and HS-5 cells were mixed in a 1:1 ratio. After the spheroid formation, an irregular, tissue-like morphology was observed, unlike the round spheroids seen in the monoculture models. On using red and green fluorescent cell trackers for staining the K562 and HS-5 cells, respectively, it was observed that there were regions of yellow fluorescence (seen as white in **Figure 5-4**). This indicated that the cancer cells and fibroblasts were visibly connected during spheroid formation.

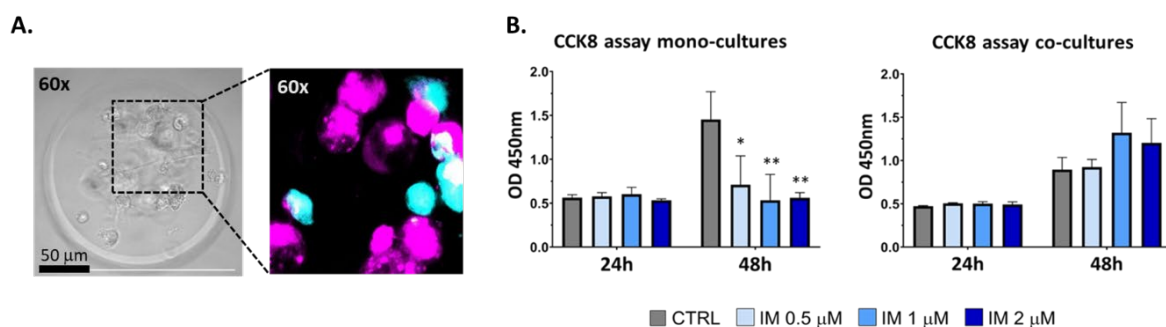


Figure 5-4: Effect on drug resistance in leukaemia cells in microdroplets. **A.** Co-culture of leukemic cells K562 (magenta) and HS-5 fibroblasts (cyan) in GelMA microdroplet with visible interconnections (white) [artificial colouring; original cells stained with CMTPX (red) and CMFDA (green) tracker for the K562 and HS-5 cells, respectively]. **B.** Graphs showing the development of resistance to Imatinib treatment with higher viability in co-cultures compared to monocultures.

To check if such interactions had any implications in the growth of the cancer cells, drug resistance analysis with Imatinib was performed in the monoculture vs the co-culture models¹⁸¹. In general, it was observed that the cell growth rate was slower in co-culture models than in monoculture models (**Figure 5-4**). However, in the co-culture models, the drug failed to arrest cell proliferation, unlike in the monoculture models.

2.2 Developing 3D models for breast cancer

2.2.1 Monoculture model

The triple negative breast cancer cells, MDA-MB-231, were grown in the three hydrogel compositions: GelMA 4.8%, GelMA 4.8% with 0.4% HAMA, and GelMA 4.8% with 0.4% HAMA and 0.4% ECM (results from cell growth and proliferation shown in **Figure 3-12**). The droplets produced with HAMA and ECM showed much better clustering of cells compared to native GelMA. A proper spheroid formation was observed only in the HAMA+ECM-containing sample (**Figure 5-5.A**). Cell aggregate area was measured by image analysis sample where the GelMA-HAMA-ECM sample had the maximum area (**Figure 5-5.B**).

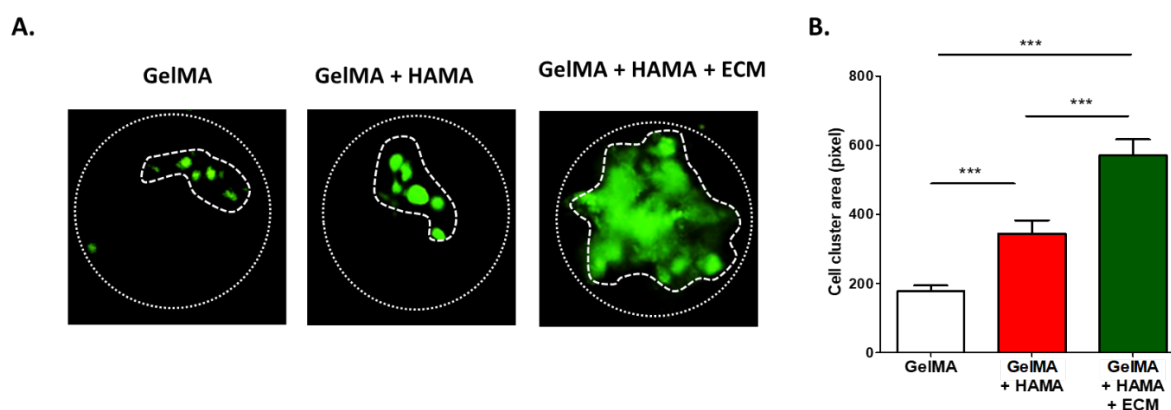


Figure 5-5: Spheroid formation in breast cancer monoculture models. **A.** MDA-MB-231 cells forming cell clusters or spheroids in encapsulation after Day 5 of culture. **B.** Area under the encircled region from figure A.

2.2.2 Co-culture model with droplets-in-droplet system

A co-culture model was attempted with the MDA-MB-231 cells in the “core” and hDFs in the “shell” to act as a disease model for breast cancer. The MDA-MB-231 cells were stained with the red cell tracking dye, CMTPX, and encapsulated in 4.8% GelMA with 0.4% HAMA to form the “cores”. These were then mixed with 10% GelMA with 1% HAMA (finally 5% GelMA with 0.5% HAMA) pre-mixed with CMFDA-stained hDFs to perform the re-encapsulation. It was observed that rigorous mixing could damage the cores, which occurred for the first sample at a flow rate (Q_H-Q_O): 8-8 $\mu\text{L}/\text{min}$ and was carefully avoided for the next sample at a flow rate(Q_H-Q_O): 30-120 $\mu\text{L}/\text{min}$ (optimizations from Chapter 4).

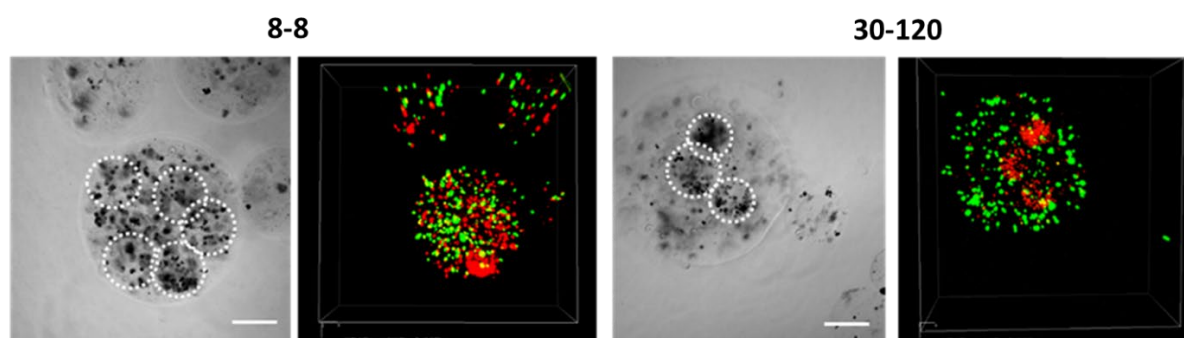


Figure 5-6: Breast cancer cells (MDA-MB-231) labelled with CMTPX dye (red) encapsulated in the core and human dermal fibroblasts (hDF) labelled with CMFDA dye (green) in the shell observed at Day 4 after re-encapsulation (Scale bar = 200 μm).

The addition of HAMA to the system decreased the migration of the cells from the droplets without preventing cell clustering. The cores and shells retained their structure for a week of culture. After Day 3, migration of cells from the core to the shell could be observed.

2.3 Drug testing in complex co-culture models

The droplet-in-droplet system was tested for application in drug testing to check if drug molecules penetrate into the cells in the “core”. In the 4.8% GelMA cores, HeLa-GFP (green fluorescent) cells were encapsulated, while RFP-hDFs (red fluorescent) were encapsulated in the 5% GelMA+ 0.5% HAMA shells. Different concentrations of rhodamine-conjugated doxorubicin were added to the core-shell droplets in the media.

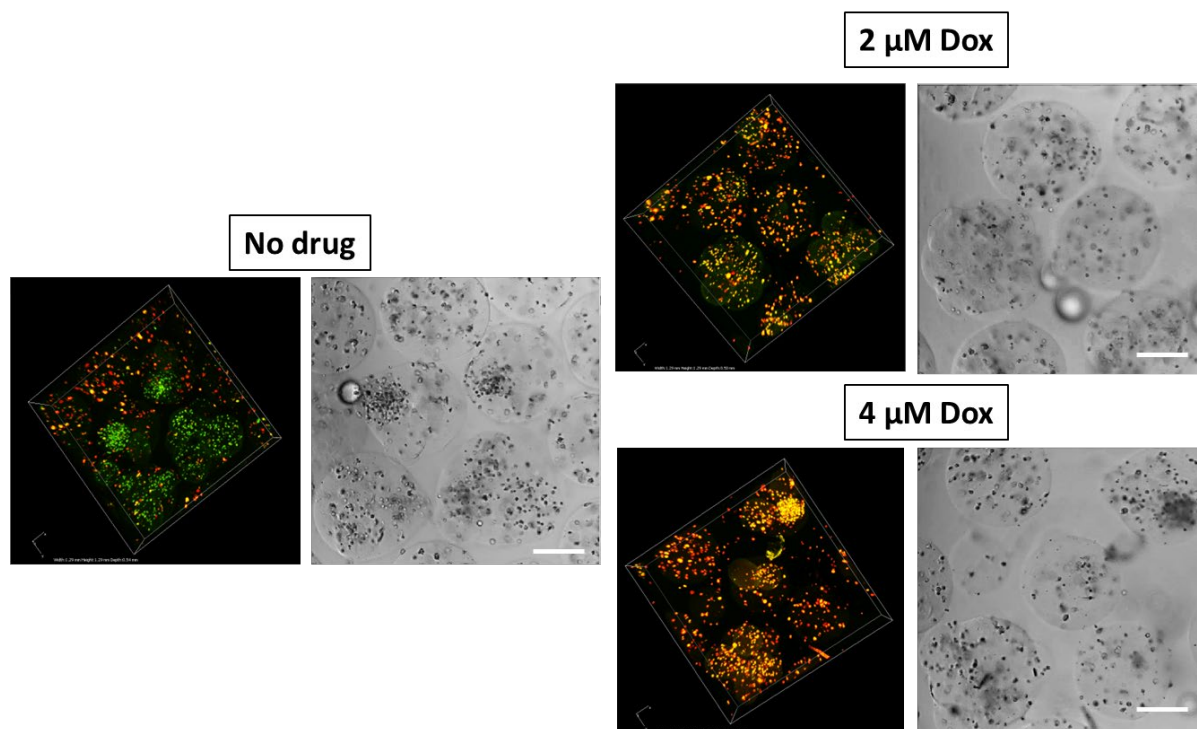


Figure 5-7: Drug testing in re-encapsulation system. Different doses of rhodamine-conjugated doxorubicin added to core-shell droplets with HeLa-GFP (green) in the core and RFP-hDFs (red) in the shell show penetration of the drug into the core cells after day 1 of treatment.

The HeLa-GFP cells initially showed green fluorescence before the addition of the drug. Depending on the concentration of Doxorubicin administered, the cells in the core turned orange/yellow as the rhodamine-conjugated drug penetrated into the HeLa cells after day 1 of drug administration (**Figure 5-7**). The control setup without the drug retained its green fluorescence. This showed that this re-encapsulation system was capable of applications in drug testing. The system can be explored for other potential applications in the future.

3 Discussion

[Tumour models demonstrated in droplets and other models with degree of complexity]

Different droplet microfluidics-based methods have been used for generating 3D tumour models previously. In a recent study, PEG and gelatin with maleimide moieties (GelMAL; instead of methacrylate) were used for making glioblastoma models by Payan and colleagues¹⁸². These models were further used for testing different concentrations of the drug temozolomide (TMZ). A high-throughput droplet generation system such as ours was demonstrated by Seeto *et al* to formulate microdroplets with high cell concentration in individual droplets to model breast cancer¹⁸³. Invasive types (MDA-MB-231) were compared to non-invasive types (MCF-7), and the former models were tested with the drug doxorubicin at high concentration (100 μ m). These breast cancer cells were also used to make micro-encapsulation models with alginate-gelatin using a Büchi encapsulator device by Ertekin and colleagues¹⁸⁴. Endothelial cells (EA.hy926) were also encapsulated in these microcapsules to compare tumourigenicity, cell migration, and drug resistance. Wu and colleagues encapsulated

colon cancer cells (HCT-116) in GelMA droplets and demonstrated co-culture models with fibroblasts to show differences in spheroid formation in mono vs co-cultures, similar to our results¹⁸⁵. They further improved their models to connect them to a vessel-on-chip device and demonstrated higher drug resistance in this tumour-TME model. A core-shell breast cancer model (with MDA-MB-231 cells) was developed by Li *et al* to mimic cell-ECM interactions¹⁸⁶. The core with the cells comprised of Matrigel or collagen I or alginate, while the shell was made of alginate with Ca-EDTA for crosslinking.

Apart from the above-mentioned models in droplets, models for metastatic prostate cancer were prepared from GelMA-HAMA using superhydrophobic surfaces in-air¹⁸⁷. Co-culture models were generated with osteoblasts integrated into this system to check for mineralization and drug resistance. A water-in-oil emulsion technique, PEG with fibrinogen, was used to form microspheres with two photoinitiators by Pradhan and colleagues¹⁸⁸. MCF-7 cells grown in the microspheres were compared to spheroids formed by the hanging drop method, showing differences in terms of tumour progression and malignancy. There have been reports of non-droplet-based cancer models in gelatin-based hydrogels using microwells^{189,190}. Breast cancer models have been developed using such systems, where spheroid formations have been demonstrated. These 3D models in hydrogels are even superior to scaffold-free spheroids due to the impact of the ECM-like hydrogels¹⁹¹.

[Effect of 3D culture in cancer cells: spheroid formation]

In the cancer models mentioned above, including ours, spheroid formation by the encapsulated cells has remained a significant observation. The spheroids are essentially 3D cell aggregates that mimic the features of small avascular tumours, such as nutrients and oxygen gradients, proliferation, cell–cell and cell–ECM interactions and also heterogeneity. The cancer cells follow certain mechanisms to generate these spheroids:

- **Initial aggregation/cell–cell adhesion:** Cells within hydrogels or in adhesion-competent scaffolds first begin to contact each other via cell adhesion molecules such as cadherins. The interactions are faster if cell–matrix adhesion is low, which can be modulated or in scaffold materials with adhesion motifs (e.g. gelatin, collagen, HA) through CD44 expression. HA has been shown to promote CD44 expression and reduce E-cadherin for the formation of hepatocellular carcinoma spheroids¹⁹².
- **Matrix stiffness, mechanical cues and external forces:** Cells sense stiffness via mechanotransduction pathways (integrin / focal adhesion/cytoskeleton), which regulate proliferation, gene expression, and motility. These can restrict the expansion of cells in stiffer hydrogels due to higher mechanical stresses from the matrix, while larger spheroids can be formed in soft hydrogels due to easier degradability/remodelling of the matrix^{193,194}. Physical forces (compression, perfusion/flow, interstitial flow), cyclic loading or deformation, also influence spheroid shape, growth dynamics, invasion behaviour¹⁹⁵.
- **Matrix properties and mass transport:** As spheroids grow beyond a certain size, the interior becomes deprived of oxygen/nutrients; cells in the core may become quiescent or necrotic; diffusion limits also influence the growth rate and shape¹⁹⁶. The scaffold's permeability, porosity, and degradation affect those gradients¹⁹⁷. For cancer models used in drug testing, the matrix properties also determine gradients of metabolites/drugs reaching the cell, thereby affecting drug sensitivity.

- **Cell-matrix adhesion and signalling:** Cells interact with the hydrogel matrix via integrins or other receptor–ligand interactions; some hydrogels supply adhesion ligands such as RGD motifs and CD44 ligands, others do not. Integrins bind ECM ligands, transmit mechanical signals to the cytoskeleton and activate downstream pathways (FAK, MAPK, etc.), which influence cell survival, proliferation, stemness, motility^{192,198}.
- **ECM remodelling by cells:** Cells secrete matrix remodelling enzymes (MMPs, etc.), exert contractile/tensile forces on the matrix, reorganise the ECM fibres, degrade or remodel parts of the scaffold / ECM (by enzymatic or hydrolytic methods) to make space, align fibres, generate mechanical feedback^{199,200}. This supports invasion (once spheroid forms) or expansion.

[Advantages and drawbacks of our models and future perspectives]

The cancer models that were produced by us had several advantages, such as rapid and high-throughput production. It has higher physiological relevance compared to 2D models for studying spatial orientation, growth mechanisms and dynamics, and resistance to drugs while mimicking cell-cell and cell-ECM interactions. Our models could be used to culture different types of cancer cells. The multicellular microdroplets allowed faster spheroid formation. The microfluidic system allowed us to make tens of thousands of droplets, providing a large population of droplets for drug testing applications, making production of such 3D models time-efficient and easy to fabricate.

However, the current model has some limitations of its own. The number of cells per droplet varies due to the flow dynamics, hence the size of spheroids (or number) varies from droplet to droplet. This can be improved by methods to break cell aggregates during the droplet production to allow better cell distribution or by cell actuation to make sure that each droplet encapsulates an equal number of cells. Furthermore, the soft hydrogels are very easily degradable by enzymes like MMPs, resulting in rapid cell migration, making the modelling of very aggressive cancers difficult in our system. This can be modified by adding hydrogels like PEG as a shell. Also, the models are currently quite simple, thereby still not being fully micro-versions of the original tumours. The models could be made more complex by introducing immune cells or stem cells to enhance their physiological relevance.

Chapter 6 : Type 1 Diabetes and Encapsulation of β -cells as a Potential Treatment Option

Type 1 diabetes (T1D), even though less common than its counterpart, Type 2 diabetes (T2D), still affects a significant part of the population. Since it is a more common disease, most of the treatment options currently available for diabetes in general are focused on T2D, resulting in inadequate therapeutic options for T1D. In this chapter, T1D has been discussed to enable understanding of the disease and the current challenges in its treatment. It has been shown how microfluidics has been utilized to provide a potential solution to the problem through encapsulation of pancreatic β -cells in hydrogel droplets.

History of Type I Diabetes

The term “Diabetes” originates from Greek, which translates to the word Siphon and means passing through. The word was used for the very first time by Apollonius of Memphis in the 3rd century BC (250-300 B.C.E.)²⁰¹. Though it is more commonly associated with the condition *Diabetes mellitus* (DM), where a person passes glucose in their urine (the Latin word *mellitus* means sweet), another condition called *diabetes insipidus* (DI) (*insipidus* means flavourless in Latin) also shares the same term “diabetes”. However, the latter condition has nothing to do with blood glucose, and people suffering from diabetes insipidus fail to retain water due to defects in vasopressin hormone production from pituitary glands (central DI) or kidneys (nephrotic DI) and thereby need to urinate frequently (polyuria) and thereby feel excessively thirsty frequently (polydipsia)²⁰². These two symptoms, however, are common for both diabetes *mellitus* and *insipidus*²⁰³.

Reported as early as the Egyptian civilization, DM has also been talked about in contemporary civilizations in Greece and India. In India, the presence of sugar in the urine of such patients was indicated by ants in the early days. The disease was termed *Madhumeha* in Sanskrit (in the early 6th century BC) by the eminent medical practitioner Sushruta in the Ayurvedic texts²⁰⁴. The word “Madhu” in Sanskrit means honey, and “meha” means urine. Similar nomenclature was developed in other Asian nations later. In 1025, Avicenna (Ibn Sina) published his medical encyclopaedia titled “The Canon of Medicine” (*Qânun dar Teb* in Persian), where he described other symptoms of diabetes mellitus and also for the first time documented the differences between DM and DI. The role of the pancreas in diabetes was discovered by Joseph von Mering and Oskar Minkowski in 1889, followed by the role of insulin in causing the disease by Sir Edward Albert Sharpey-Schafer²⁰⁵.

Causes and epidemiology

Type 1 Diabetes was more popularly known as Juvenile Diabetes and was considered to only affect children until very recently. Though the major symptom of hyperglycaemia (high blood glucose/sugar) is common between both Type 2 and Type 1 diabetes, the factor causing this increase in blood glucose levels is drastically different. While insulin insufficiency results in Type 2 diabetes, Type 1 diabetes is a result of an inadequate number of beta cells in the islets of Langerhans in the pancreas. This loss of beta cells is mostly attributed to their destruction by auto-antibodies within the patient’s system, which identify the beta cells as foreign bodies.

Based on the type of pathogenesis, T1D can be classified into antibody-positive (1A) and antibody-negative (1B) types. These auto-antibodies may develop due to genetic factors or can also be a result of a viral infection in the patient. It has also been observed that ethnicity and environmental factors have some influence on T1D pathology.

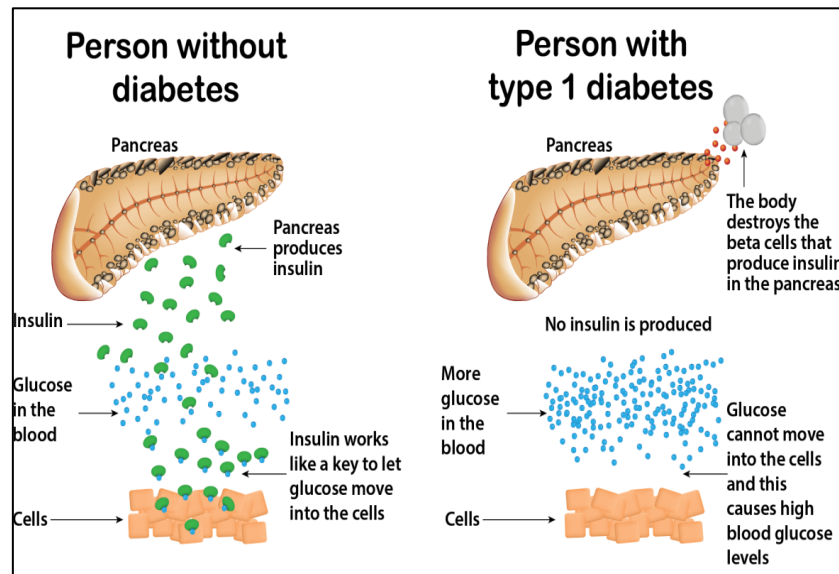


Figure 6-1: Pancreatic function in a normal person compared to a person suffering from Type 1 Diabetes.

Analyzing the immunological responses resulting in T1D revealed a significant role of T-helper (Th) cells and regulatory T cells (T_{reg})²⁰⁶. Th1 and Th17 cells produce proinflammatory cytokines such as interleukins (IL- 2/17/21/22), interferon- γ (IFN- γ), and others, which interfere with the immune regulation in the β -cell microenvironment. This is mostly attributed to the imbalance in Th17 to T_{reg} levels. Even poorly developed or partially functional T_{reg} cells fail to control the effector T cells and lead to autoimmune responses, leading to T1D. Other than these $CD4^+$ T-cells, $CD8^+$ T-cells have also been associated with T1D.

Other specific types of T1D include fulminant type 1 diabetes (FT1D), which was initially known as idiopathic T1D, where rapid and complete destruction of β -cells takes place in the patient, leading to sudden hyperglycemia and severe metabolic disorders (ketoacidosis)²⁰⁷. A slow progressive subtype of T1D is the latent autoimmune diabetes of adults (LADA), where autoimmune factors, along with lifestyle factors, result in autoantibody generation against the β -cells²⁰⁸. Adults (above 30 years of age) suffering from this condition are often misdiagnosed as T2D patients, however, insulin supplementation is mostly not required up to 6 months after the onset of the condition

Focusing on the genetic factors, most of the dysfunctional genes affecting beta-cell signalling and apoptotic pathways result in T1D, like *CTSH*, *ERBB3*, *GLIS3*, and *HIP14*²⁰⁹. Some of the genes, like *IFIH1* and *PTPN2*, with *BACH2* are altered due to interactions with viral dsRNA during infections. The most significant genes, however, include the different haplotypes of the HLA-class II genes located on chromosome 6p21, namely *HLA-DRB1*, *HLA-DQB1/DQA1*, and *HLA-DPB1/DPA1*. In later studies, associations were also found with the HLA class I genes, which are associated with T-cell repertoire and antigen-specific

cytotoxicity, for genetic predisposition to Type-I diabetes and provides an immunologic explanation.

Current Treatment Options and Challenges

The treatment options that are currently available for Type 1 diabetes majorly coincide with the Type 2 diabetes treatments available due to similarities in symptoms, as previously mentioned. The most commonly followed treatment is insulin supplementation through intramuscular injections. The first insulin injection treatment was performed on a 14-year old boy from Toronto named Leonard Thompson in 1922²¹⁰. However, this treatment option is quite superficial and, in several instances, fails to meet the increasing requirement of insulin in the patient's body due to the continuous loss of beta cells. Additionally, since most of the T1D patients are children, compliance with regular administration of these injections is quite difficult in most cases.

A much more targeted treatment option is the transplantation of functional pancreatic islets to the patients. However, transplantation has several associated risks, such as rejection by the patient's system even after donor matching, which can even lead to death. Also, it is difficult to find suitable deceased donors to obtain such islets. Moreover, the transplantation process needs an invasive surgery, which has post-operative complications like septicaemia, hypersensitivity, bleeding from sutures, delayed recovery, and others. None of these therapeutic options can provide protection from the auto-antibodies generated within the patient's system, which can still continue to act against the beta cells present.

Among other therapeutic strategies currently under research, anti-CD-3 monoclonal antibodies have been shown to decrease T-cell immunogenicity by decreasing T effector cell levels while increasing the functionality of T_{reg} cells²¹¹. To simplify the production and administration of such treatments to the patients, microfluidics can play an important role.

In this chapter, rat pancreatic β -cells (RIN-m) or human pancreatic islet cells (hPICs) were encapsulated, and aspects of their long-term growth, proliferation, and function were analysed. Firstly, rat insulinoma (RIN-m) cells were used as model cells for checking droplet production and cell behaviour, and physiology in different hydrogel compositions based on the previous optimizations. The cells were cultured in the droplets for up to two weeks, and glucose susceptibility tests were performed. The selected hydrogel composition was used for encapsulating human pancreatic islet cells. The encapsulated cells were tested for various parameters such as DNA damage (H2AX assay) and oxidative stress (ROS assay). The droplet stability was checked in long-term culture for more than two weeks.

1 Materials and Methods

1.1 Cell culture

Rat insulinoma (RIN-m) cells were cultured in a T75 tissue culture flask in complete RPMI-1640 media (Gibco, Thermofisher, US). Cells above passage 10 were taken for experiments.

Human pancreatic islet cells (CliniSciences, Poland) were grown in human pancreatic ECM-coated T75 flasks in complete islet cell media and taken for experiments after passage 2.

1.2 Cell encapsulation experiments

The RIN-m or human pancreatic cells were mixed in media at a concentration of 20 million/mL. This cell stock was mixed in a 1:4 ratio with hydrogel pre-polymers. The droplets were generated in the presence of HFE-7500 (Novec) using the circular chip generation method with on-the-fly or stationary crosslinking with 15 seconds of exposure to 385 nm UV ($Q_H = 25 \mu\text{L}/\text{min}$, $Q_O = 125 \mu\text{L}/\text{min}$). The generated crosslinked droplets were collected and washed with 20% PFO in Novec, followed by rinsing with pure Novec. Finally, the droplets were washed thrice in PBS using centrifugation for 5 minutes at 1200 rpm to remove the oil completely.

1.3 Cell viability and proliferation assay

Cell viability of the encapsulated cells was analysed using the Live-dead viability assay kit (Thermofisher GmbH, Germany) in a ratio of 30 μL in 200 μL of microdroplets in PBS. The live cells were detected using a 488 nm laser (green fluorescent) while the dead (or membrane-damaged) cells were detected using a 560 nm laser (red fluorescent).

For proliferation, the CCK-8 assay was performed. To 90 μL of cells/droplets, 10 μL of the CCK-8 dye was added and incubated in the cell culture incubator for 3-4 hours and then a UV spectrophotometer was used to detect the absorbance values indicating metabolically active cells. The cells were monitored over two weeks (Day 1, 3, 5, 7 and 14).

1.4 LDH cytotoxicity assay

The CyQUANT™ LDH Cytotoxicity Assay Kit (Thermofisher GmbH, Germany) was used according to the protocol provided with the kit. Briefly, cells were cultured in the incubator in wells of the 96 well plate and each set treated differently (i) a small amount of deionized water was added during incubation (for Spontaneous LDH), (ii) normal incubation but lysis buffer was added prior to testing (for Maximum LDH), and (iii) normal incubation and treatment of samples until the test was performed. For the test, 50 μL of media was taken from each of the wells and to it 50 μL of the assay substrate was added and incubated for 30 minutes at room temperature in the dark. The absorbance was read at 490 nm and 680 nm (background) after adding 50 μL of the stop solution to each well.

1.5 Morphology Analysis

Morphology of encapsulated cells was analysed using Phalloidin for staining the cytoskeleton of the cells and DAPI for staining the nucleus. For staining the droplets with cells, they were fixed using the paraformaldehyde (PFA) fixation protocol. Firstly, droplets with encapsulated cells were washed with cold PBS thrice by centrifuging at 1200 rpm for 5 minutes each. After the final wash, the PBS was removed, and the droplets were resuspended in 200 μL of 4% PFA and added to a coverslip placed in a 2.5 cm petri dish over ice for 20 minutes. After this, the residual PFA was carefully removed from the edges of the petri dish, and the cover

slip was slowly washed with 500 μ L of PBS thrice without detaching the droplets on the coverslip. A 200 μ L of 0.05% Triton-X was added to the droplets and incubated for 10 minutes and incubated at room temperature. The samples were washed slowly with PBS three times before adding a 2% BSA solution for 1 hour for blocking at room temperature (or overnight at 4°C). After an hour, the BSA solution was removed and washed once with PBS before adding 200 μ L of Phalloidin stain for 20 minutes at room temperature. After 20 minutes, the stain was removed and washed with PBS twice and similarly stained with DAPI for 20 minutes. Finally, two times washing was done and the cover slip was kept in PBS solution until microscopic observation (to prevent drying out of the droplets).

1.6 DNA damage assay (H2AX)

For checking the DNA damage due to our encapsulation process, a H2AX antibody-based assay was performed in the encapsulated cells. The droplets with cells (also 2D cultured cells) were fixed and permeabilized according to the protocol described in the previous section (Section 1.6). After the BSA blocking step, the droplets were washed with PBS slowly, and a working solution of the antibody, Phospho-Histone H2A.X (Ser139) monoclonal antibody (3F2; Thermofisher GmbH, Germany), was made with 0.1% BSA and added to the coverslip and incubated at room temperature for 3 hours. After incubation, the cover slip was washed thrice with PBS carefully, and the secondary Anti-mouse antibody (488 nm fluorophore-labelled; Thermofisher GmbH, Germany) was added (with 0.1% BSA) and incubated for 1 hour at room temperature. Finally, the droplets were carefully and thoroughly washed with PBS and observed under the microscope.

1.7 Oxidative stress analysis

Oxidative stress was checked in the samples using a fluorescent probe named CellROX™ Orange Reagent (Invitrogen, Thermofisher GmbH, Germany). A 5 μ M concentration of the dye was added to the live cell samples and incubated at 37°C for half an hour. Counterstaining of the nucleus was done with Hoechst 33342 (Thermofisher GmbH, Germany). The cells were observed at 488 nm and 525 nm excitation ranges.

1.8 Immunocytochemistry staining

Droplets with cells were fixed as described above using PFA and permeabilized with Triton-X. The antibodies (insulin primary and glucagon primary) were diluted in a 1:10 ratio in blocking buffer (5% BSA with 0.01% Triton-X) and kept at 4°C overnight for antibody binding. The following day, the immunostaining solution was washed twice with PBS, followed by the addition of nuclear stain DAPI (2 mg/ml) and incubating at room temperature for 20 minutes. Finally, the dish/cover slip was washed with PBS twice and air-dried before observing under a confocal microscope.

1.9 Insulin production ELISA test

The droplets were cultured for two weeks with approximately 1000 droplets per well in 96-well plates. A 50 μ L of media was collected (and replaced) from each of the wells every 1, 3, 5, 7, and 14 days of culture for general insulin production analysis.

The media collected from the samples were tested using the Rat/mouse insulin ELISA kit (Merck GmbH, Germany). The test was performed according to the protocol provided in the kits. Briefly, a sandwich immunoassay was developed after adding the samples diluted with assay buffer into each of the wells. The standards provided with the kit were added to the first few wells in ascending order of concentration. The detection antibody was added after incubation and again incubated to allow attachment to the detected molecules. The enzyme and substrate solutions were sequentially added and incubated to generate a concentration-dependent blue colour, which was converted to yellow after the addition of the stop solution. Finally, the HTX synergy plate reader was used to detect the absorbance values at 490 nm. A graph was plotted for the standards with known concentrations, and the interpolated values were derived for the unknown samples to calculate the insulin concentration.

1.10 Image and statistical analysis

All fluorescent analyses were performed using the Nikon A1 confocal microscope. The images were analysed using the Fiji ImageJ software. The ROI functions were used after converting the fluorescent images into binary (and the threshold was adjusted). The number of particles, area, and integrated density of fluorescent zones were calculated. Graphical and statistical analysis was done using GraphPad Prism 5.

2 Results and Inferences

2.1 Hydrogel optimization for beta cell encapsulation

2.1.1 Cell behaviour in bulk hydrogel

For hydrogel optimization studies, RIN-m cells were used due to their fast growth and proliferation rate (since it is a cancer cell line). As tested previously in Chapter 3, the following three hydrogels were used: (i) 4.8% GelMA, (ii) 4.8% GelMA with 0.4% HAMA, and (iii) 4.8% GelMA with 0.4% HAMA and 0.4% porcine pancreatic ECM. As an initial step, the bulk hydrogel (50 μ L) was mixed with the RIN-m cells and added to a well of the 96-well plate. Similar to the earlier obtained results, the cells in GelMA-HAMA-ECM showed the maximum cell viability out of the 3 hydrogels (almost no red cells were detected; **Figure 6-2**).

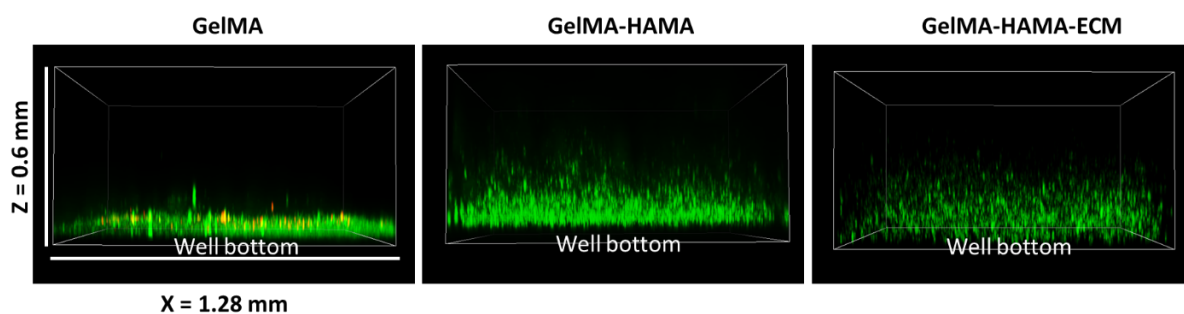


Figure 6-2: Bulk hydrogel optimization. Live (green) and dead (red) staining of RIN-m cells encapsulated in GelMA, GelMA with HAMA, and GelMA with HAMA and ECM after 3 days of culture.

The cells could easily migrate in the GelMA hydrogels primarily due to the larger pores, which were restricted in the ECM-containing hydrogel due to the interpenetrating networks of the composite. The migration can also be attributed to matrix-degrading enzymes secreted from these insulinoma cells²¹².

2.1.2 Viability analysis in the hydrogel droplets

The three hydrogel compositions were then tested for droplet formation in the OTF system at the optimized flow rates (25 $\mu\text{L}/\text{min}$ and 125 $\mu\text{L}/\text{min}$, respectively, for the hydrogel phase and the continuous phase). The droplet generation process remained quite smooth, as for the other cells. The viability of the encapsulated cells remained above 75% for all the hydrogels (**Figure 6-3.A.**). From the image analysis, the GelMA-HAMA-ECM droplets showed maximum cell viability with an average of 85%.

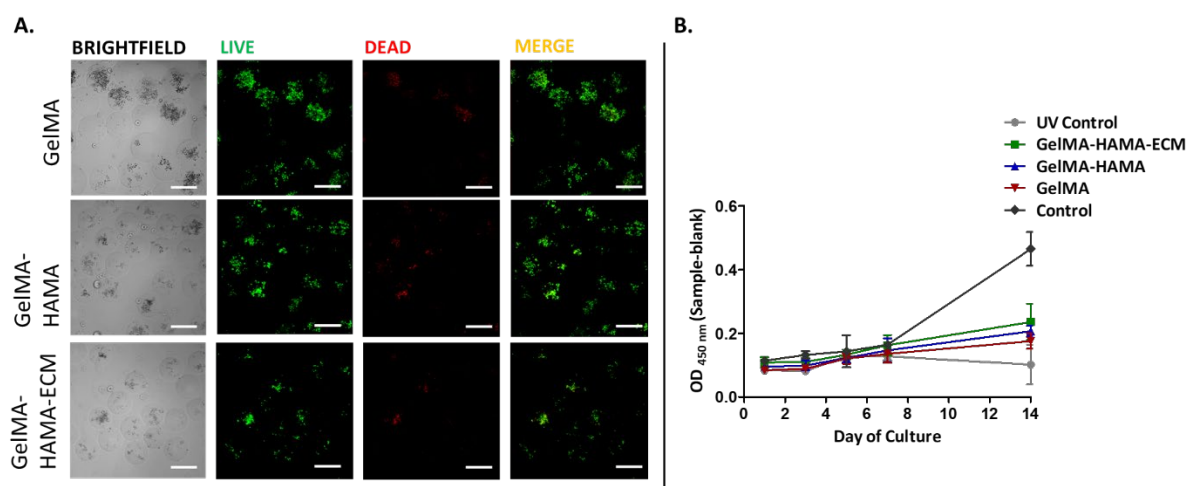


Figure 6-3: Viability analysis of encapsulated beta cells in droplets. **A.** Live-dead image analysis of cells encapsulated in the different hydrogels on Day 1 of culture (Scale bar = 200 μm). **B.** CCK-8 analysis of the cells in droplets compared to controls (RIN-m cells exposed to 385 nm UV or not).

The encapsulated cells were also tested using the CCK-8 assay against normal RIN-m cells and cells exposed to UV (385 nm) for 15 seconds (same as exposure time in the OTF system). Out of the three hydrogels, GelMA-HAMA-ECM droplets again showed better viability with values comparable to even control cells (without UV exposure) by Day 7 (**Figure 6-3.B**). The UV-exposed cells without any encapsulation showed poor viability with a decline

in viability over the two-week culture period. In all other cases, cell viability increased over time. However, since the space was restricted in the case of the droplets, the proliferation was restricted due to the interpenetrating pores of the composite hydrogels. The GelMA droplets mostly dissolved by Day 14, and cells migrated to the bottom of the well (**Figure 6-4. B**).

2.1.3 Morphology analysis for long-term culture

The conditions of the cells were also analysed on the basis of morphology in encapsulation. The cells could not attain their normal triangular-type morphology in encapsulation but appeared rounded instead. To check if their nucleus and cytoskeleton were intact, the cells were fixed and stained with DAPI and Phalloidin (stains F-actin filaments) on Day 7 of culture. All encapsulated cells in all hydrogels showed staining for both stains (**Figure 6-4.A.**). The cells in GelMA appeared slightly smaller, probably due to the stress from the dissolving hydrogel. However, cell clustering was observed for all 3 cases, with the GelMA-HAMA-ECM sample showing the most prominent clustering. The porcine pancreatic dECM seemed to provide a familiar microenvironment to the β -cells.

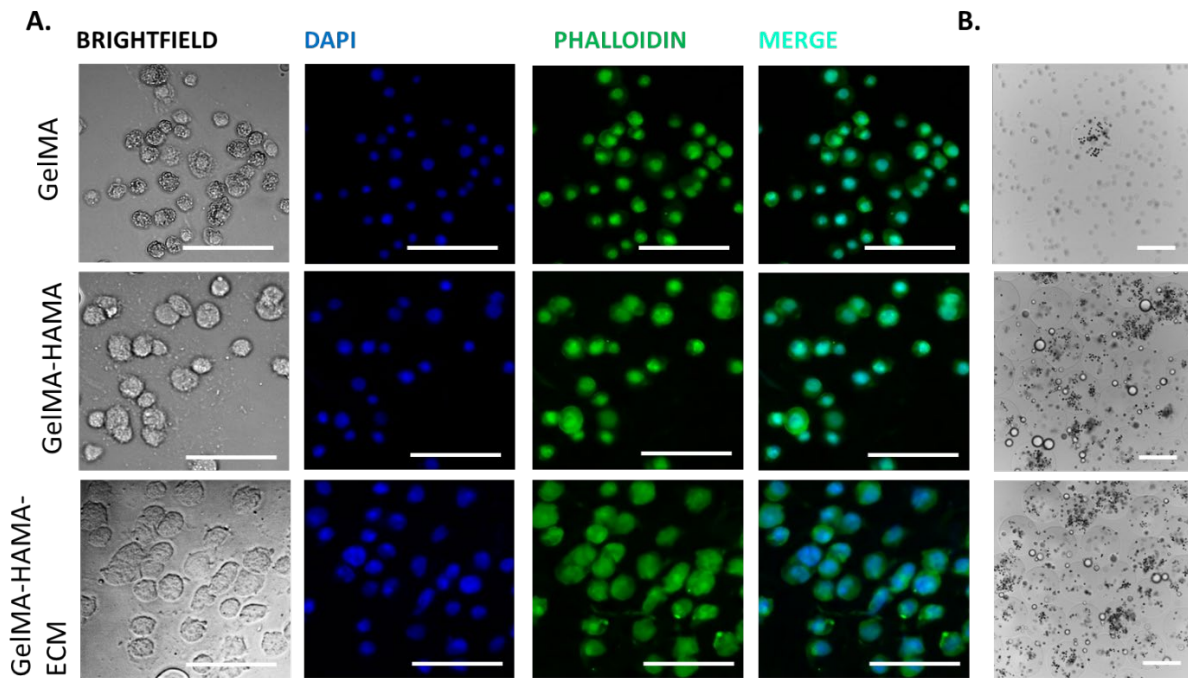


Figure 6-4: Morphology of cells in encapsulation for long-term cultures in different hydrogels. **A.** Encapsulated cells stained with DAPI (nucleus) and Phalloidin (cytoskeleton) observed under a confocal microscope (at 55x magnification) on Day 7 of culture (Scale bar = 50 μ m). **B.** Droplets with cells observed on Day 14 of culture (Scale bar = 200 μ m).

The cells in encapsulation were observed again after another week of culture. The GelMA droplets had mostly dissolved by this time, while the cells were released and settled on the well bottom (**Figure 6-4.B.**). The cells in the GelMA-HAMA and GelMA-HAMA-ECM droplets did not migrate out of the droplets. Also, unlike fibroblasts, they do not grow on the droplets. Hence, the GelMA-HAMA-ECM could be used for β -cell encapsulation purposes without problems of migration.

2.1.4 Growth rate comparison and its effect on Insulin secretion

The insulin secretion from the encapsulated cells in each of the three hydrogel compositions was tested by ELISA assay. For GelMA, the insulin production was lower on the first day of the culture but turned out to be better than the other hydrogels till Day 7, after which the growth and insulin secretion both declined as the droplets dissolved (**Figure 6-5.A.**). In the case of GelMA-HAMA, the insulin secretion also increased with culture time. For the GelMA-HAMA-ECM, the insulin secretions were consistent throughout with an average of 48 ng/mL, but it also had the maximum insulin release compared to the other two hydrogels by the end of two weeks (~58 ng/mL).

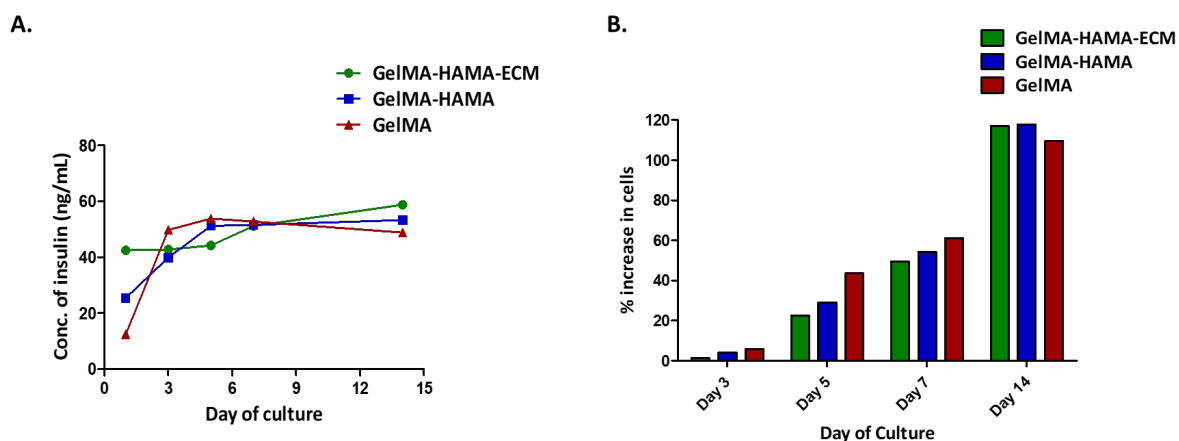


Figure 6-5: Insulin secretion and cell proliferation in long-term culture. **A.** Concentration of insulin secreted over the two weeks of culture from the ELISA assay. **B.** Proliferation of cells compared to day 1 of culture in each of the hydrogels obtained by CCK-8 analysis.

The insulin secretion pattern kind of correlated to the proliferation rate of the cells in encapsulation over time (**Figure 6-5.B**). When compared to Day 1 of culture, the proliferation rate of cells in GelMA remained the maximum for one week of culture and decreased as the proliferation rate reduced by the next week. A similar pattern can be noticed in the insulin secretion from the cells in GelMA as well. Similarly, for the GelMA-HAMA-ECM, the insulin secretion reached a maximum when the proliferation rate was the maximum among the three hydrogels after 14 days of culture. Considering all the factors, GelMA-HAMA-ECM was hence considered as the most suitable hydrogel for all future β -cell/islet cell experiments.

2.2 Wavelength Optimization for droplets

2.2.1 Viability variations in 385 nm vs 405 nm crosslinking

As mentioned previously, blue light (405 nm) has been considered a better wavelength for photocrosslinking with cells compared to UV light (<400 nm). This was checked for the β -cells encapsulated using the OTF system with the 385 nm and 405 nm lamps. The encapsulation was done in the optimized hydrogel, 4.8% GelMA with 0.4% each of HAMA and ECM. The cell viability on Day 1 of culture in case of 385 nm crosslinked droplets was slightly higher than the 405 nm crosslinked droplets (~85% for 385 nm and ~82% for 405 nm; **Figure 6-6.A.**).

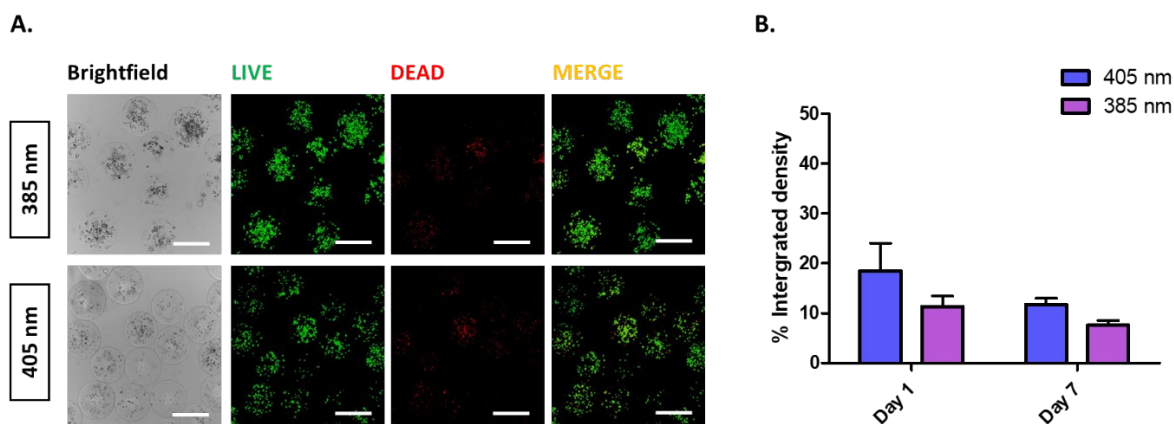


Figure 6-6: Viability of beta cells based on crosslinking wavelength. **A.** Live (green) and dead/membrane-damaged cells (red) observed under confocal microscope on Day 1 of culture. **B.** Cell recovery observed based on a decrease in integrated density (of fluorescence) over a week of culture.

On further investigation by image analysis, it was observed that slight red fluorescence was visible in several cells for both cases, indicating possible membrane damage or cellular stress (or both). The signals appeared to reduce over time (one week in culture). This was quantified based on the fluorescent signals (gray value for binary images) depending on the area covered by the cells (integrated density = Area $\times$ Mean Gray Value). The percentage of integrated density represented above in **Figure 6-6.B.** is the ratio between the red fluorescent to green fluorescent integrated density. It can be clearly observed that in both cases, the average percentage of integrated density of red fluorescence decreases over the week in culture (more prominent for 405 nm). However, the 385 nm droplets still maintain a lower percentage throughout the observation period. The reason for the cell recovery from stress is possibly due to the hydrogel composition providing a sustainable microenvironment.

2.2.2 Structure and function variations

The cells encapsulated using the crosslinking wavelengths of both 385 nm and 405 nm show no structural problems when observed after DAPI-Phalloidin staining (**Figure 6-7.A**). The nuclei in the 405 nm crosslinked structure appeared slightly larger than those in the 385 nm crosslinked droplets. This was probably due to the weaker crosslinking in the 405 nm droplets, allowing more space for the cells to spread, compared to the 385 nm droplets, which have better crosslinking, resulting in more even pore sizes. Clustering of the RIN-m cells was observed in almost a similar manner in both cases.

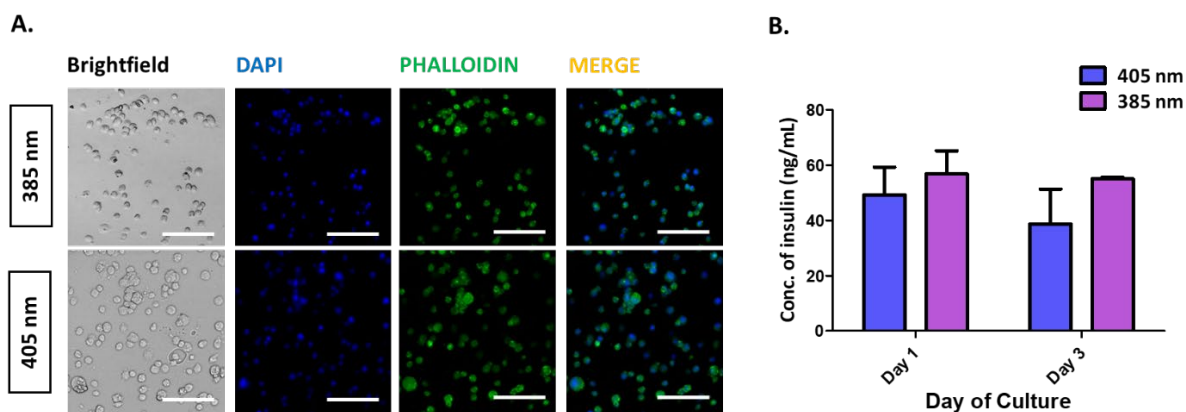


Figure 6-7: Morphology and function of beta cells dependent on crosslinking wavelength. **A.** Morphology analysis of fixed, encapsulated cells using DAPI (nucleus) and Phalloidin (cytoskeleton) staining on Day 7 of culture. **B.** Insulin secretion variations from encapsulated cells depending on crosslinking wavelength obtained from the insulin ELISA assay.

To analyse the effect of the crosslinking wavelength on the insulin production ability of the cells, it was observed that 385 nm crosslinked droplets showed higher insulin production compared to the 405 nm crosslinked droplets. This was possibly due to the complete crosslinking of the droplets at 385 nm. Also, three different experimental sets were considered for comparative analysis (indicated as the standard deviation for the error bars). For all three cases, the insulin production became consistent by the 3rd day of culture for the 385 nm crosslinked droplets. However, the insulin secretion from the 405 nm crosslinked droplets varied among the three experimental sets, mostly due to variabilities in crosslinking. Hence, the crosslinking wavelength of 385 nm was confirmed for all future experiments.

2.3 Human pancreatic islet cells in encapsulation

2.3.1 Oxidative stress in the encapsulated cells and cell proliferation

Human pancreatic islet cells were used for this set of experiments to check the effects of encapsulation in further detail. The previously optimized conditions of hydrogel composition and crosslinking wavelength were used. The method of crosslinking (OTF method or stationary method) was optimized through these experiments. The islet cells (ICs) were exposed to the 385 nm light for 15 seconds without the hydrogel to be used as a control. The other control was normal cells in 2D culture. In the cells normally grown in 2D without any UV exposure, the oxidative stress in cells is extremely low, while in the UV-exposed controls, almost all exposed cells show signs of oxidative stress (**Figure 6-8.A.**). It is interesting to observe in the encapsulation methods that in the OTF samples, even though the viscous forces are quite high, it probably does not lead to the oxidative stress seen in some of the cells, which is mostly caused by the crosslinking process. The stationary crosslinking process, even after experiencing lower viscous stresses, shows a much higher degree of oxidative stress in this case due to the irregularities of crosslinking (leading to the presence of residual free radicals) between droplets as described in Chapter 2 earlier.

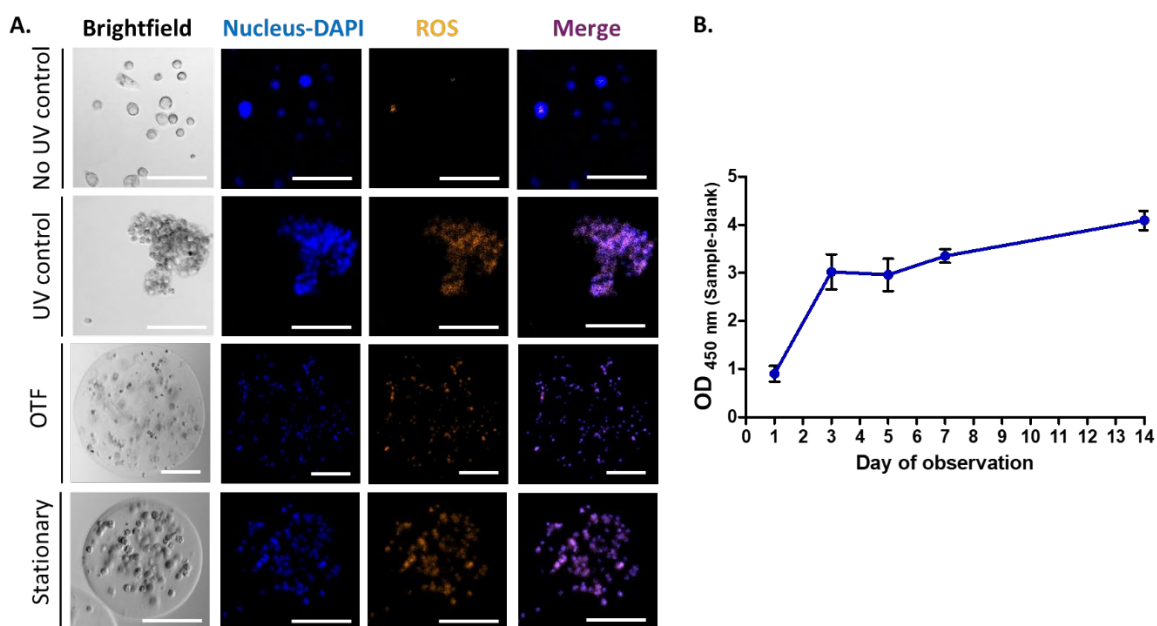


Figure 6-8: Oxidative stress and cell proliferation in ICs after encapsulation. **A.** Oxidative stress due to the encapsulation process in human ICs compared between normal cells in 2D culture (No UV control), Cells directly exposed to 385 nm (UV control), and after the two encapsulation methods on Day 1 of culture (Scale bar = 50 μ m (for controls) and 100 μ m (for encapsulation)). **B.** CCK-8 analysis of the cells encapsulated by the OTF method to check for growth over two weeks in culture.

In terms of proliferation, the cells remained highly proliferative till Day 7, and then the growth rate slowed down, but proliferation was still visible **Figure 6-8. B.**). From day 1 to day 3, the proliferation rate was quite high, possibly due to recovery from the initial oxidative stress. In general, the cells remained metabolically active and proliferative throughout their two-week culture period.

2.3.2 DNA damage in the cells due to the encapsulation process

The samples were checked for DNA damage using the γ -H2AX immunofluorescence assay. During double-stranded DNA breaks, the serine 139 at the carboxy terminus of the histone protein H2AX gets phosphorylated, thereby producing γ -H2AX. This can be detected with an antibody and correlated to DNA damage. For our experiments, we used a fluorescently labelled (green) secondary antibody to detect binding of the γ -H2AX antibody. For the UV-exposed controls, the maximum amount of DNA damage was observed among the tested samples, with most of the cells with H2AX signals covering >50% of the nucleus (DAPI-stained; **Figure 6-9.A.**). In the stationary crosslinked samples, too high a degree of DNA damage can be observed. Though lesser than the UV controls, the presence of some cells with more than half the nucleus indicating DNA damage can be noticed.

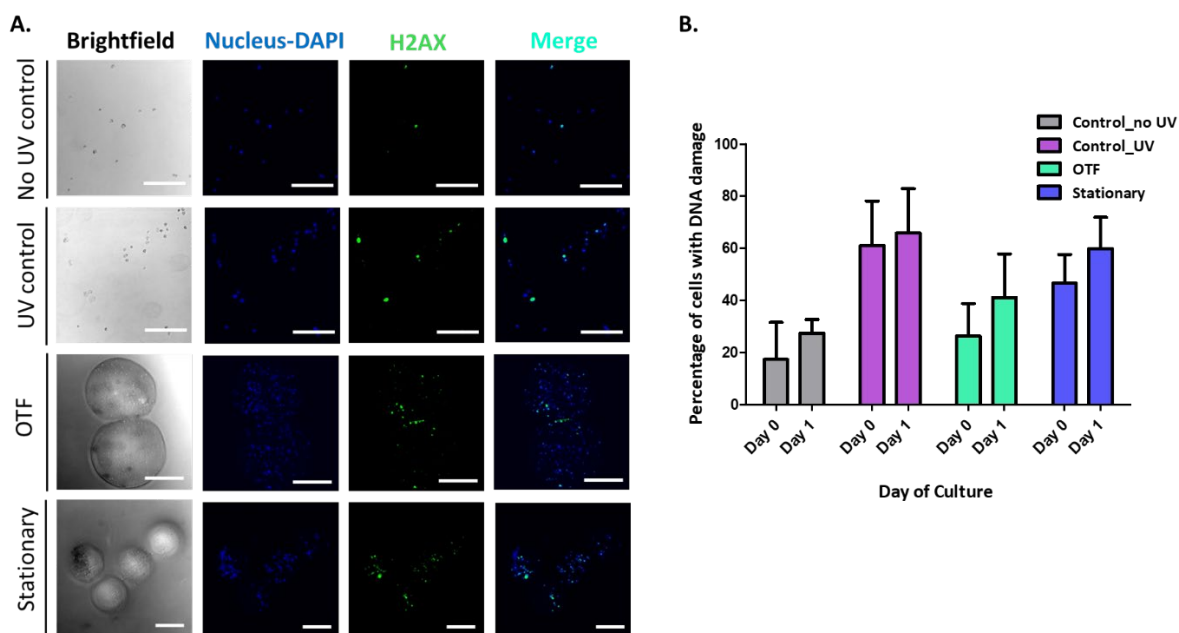


Figure 6-9: DNA damage analysis using gamma-H2AX antibody. **A.** ICs in 2D or encapsulated in GelMA-HAMA-ECM droplets showing presence of DNA damage by attachment of the H2AX antibody (green) in DAPI-stained nuclei of cells (blue) (Scale bar = 50 μ m (for controls) and 200 μ m (for encapsulation)). **B.** DNA damage was compared between the day of the experiment and after 1 day of culture.

On comparing the cells 3 hours after the treatment and after 1 day in culture, it was observed that the degree of DNA damage increases for all tested samples, even in normal 2D culture (**Figure 6-9.B.**). This indicates that double-stranded breaks are also a natural phenomenon in the ICs. For the UV-treated controls, the cells were severely damaged after exposure to UV, and most of them failed to attach to the well surface. For day 1 of culture, very few cells were available for analysis. Even the few attached cells showed the presence of double-stranded DNA breaks. The OTF samples showed a much lower percentage (<50%) of DNA-damaged cells compared to the stationary crosslinking method. Out of the cells showing DNA damage, the nuclear area of damage in OTF crosslinked cells was much lower compared to the stationary crosslinked cells or the UV controls. This also indicates that the hydrogel is capable of providing some protection against UV-mediated DNA damage during the encapsulation process.

2.3.3 Structure and function of the islet cells in encapsulation

From the previous tests, it was quite clearly understood that the OTF system was much better for the ICs in terms of lower DNA damage and oxidative stress. The cells were further checked for analysis of structural integrity, and whether the DNA damage or oxidative stress could affect the structure and function of the cells. Firstly, DAPI-Phalloidin staining was done for the encapsulated cells in the OTF crosslinked droplets. Since the ICs are a mixed population of cells extracted from the human pancreas it was observed that some of the cells behaved like fibroblasts and preferred growing on the droplet surface rather than inside them (**Figure 6-10.A.**).

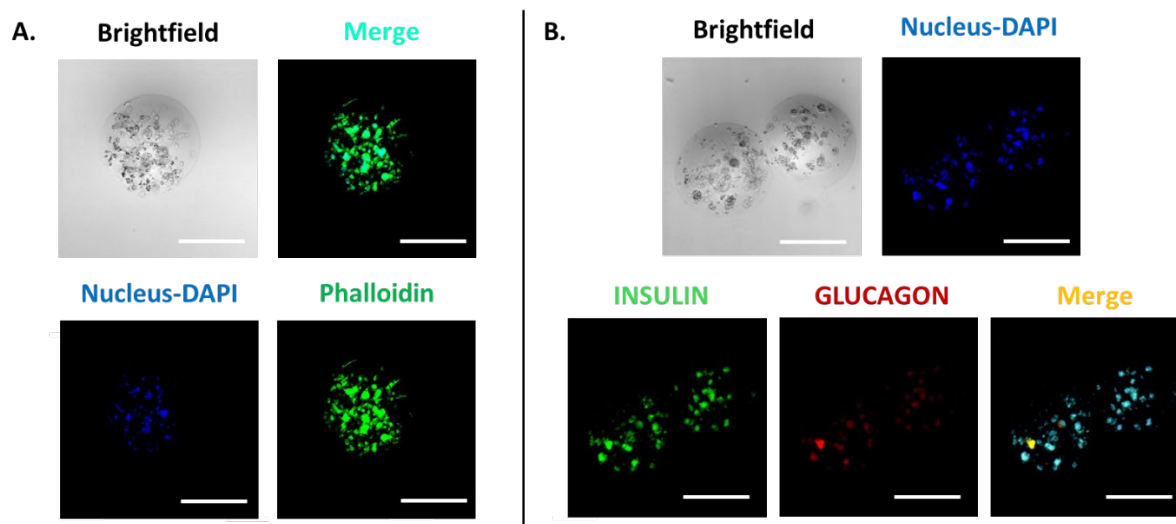


Figure 6-10: Structural and functional analysis of ICs encapsulated in GelMA-HAMA-ECM droplets by OTF crosslinking. **A.** The visible cytoskeleton (Phalloidin-stained; green) and nuclei (DAPI-stained; blue) were observed in fixed, encapsulated cells. **B.** Immunocytochemistry of the droplets shows the presence of several insulin-positive cells (green) with a few glucagon-positive cells (red) among the encapsulated cells (Scale bar = 200 μm).

The encapsulated cells were further checked for cellular function preservation by immunocytochemistry. Insulin and glucagon antibodies were used to detect the presence of cells capable of secreting each of the hormones. As is known, the β -cells (or insulin-producing cells) are most abundant, followed by a lower percentage of glucagon-producing α -cells in the native pancreatic islets. A similar pattern was visible for our ICs encapsulated in the droplets, showing a large population of insulin-positive cells and also a smaller population of glucagon-producing cells (**Figure 6-10.B.**). This proves that the low degree of DNA damage and oxidative stress experienced by the cells during the encapsulation process initially does not affect the structural and functional capabilities of the cells in the long-term culture.

3 Discussion

[Beta cell encapsulation in droplets]

In microfluidics, pancreatic β -cell encapsulation has been investigated in several studies. One of the latest studies in this field was performed by Yan and colleagues using mucin microdroplets (also compared to alginate)²¹³. The β -cells encapsulated in mucin microdroplets not only allowed the formation of islet-like structures with MIN-6 cells but also helped in immunomodulation to protect the cells from autoantibodies. A similar study was performed earlier in alginate microdroplets for MIN-6 cells, but the process had a lower throughput²¹⁴. A microfluidic flow-focusing device was used to generate β -cell-laden microgels of tyramine-conjugated PEG to provide immunoprotection²¹⁵. The droplets also facilitated the restoration of normal glucose levels in streptozotocin-induced diabetic mice.

[Beta cell encapsulation in different hydrogels]

Other than microdroplets, hydrogel-based β -cell encapsulation was also attempted in alginate microfibres, which were found to re-stabilize glucose levels after implanting in mice²¹⁶. In another study, a thiolated EphA5-Fc receptor and ephrinA5-Fc ligand were conjugated to PEG hydrogels to enhance cell-cell communication among encapsulated β -cells²¹⁷. Similarly, anti-inflammatory PEG hydrogels were synthesized by chemical ligation, which provided protection to encapsulated β -cells against cytokines.

[Role of pancreatic ECM for beta cells development]

The ECM forms an integral part of the β -cells' microenvironment and enhances the functioning and survival of these cells by providing nutrition and facilitating oxygenation. The major components of the islet cells' ECM include collagens, fibronectin, and laminin. Incorporating some or all of these components has proven to reduce apoptosis in islets encapsulated in PEG hydrogels post-transplantation *in vivo*²¹⁸. The authors also demonstrated the role of ECM-derived whole proteins, such as laminin, in enhancing insulin secretion from encapsulated β -cells compared to its derived peptides in PEGDM hydrogels. In another study, a synthetic protein-based polymer with integrated ECM components showed much better functionality of encapsulated MIN-6 cells compared to the hydrogel without the ECM components²¹⁹.

[Decellularized ECM for pancreatic β -cell encapsulation]

In a recent study, alginate with human pancreatic dECM was used to encapsulate pancreatic islets for long periods (up to 58 days) with good sensitivity to glucose susceptibility²²⁰. Alginate hydrogels have also been developed with rat pancreatic dECM and adipose tissue-derived dECM for β -cell encapsulation (with rat-MSCs in the former case), resulting in better functionality of the cells^{221,222}. The ECM-derived hydrogel called Matrigel[®] has been used to support β -cells and has been delivered alongside liposomal clodronate to allow β -cell survival for longer periods and provide immunoprotection. This was used as a thermosensitive injectable therapy²²³. Decellularized pancreatic ECM has been used as a scaffold material for infusion of pancreatic islets and INS-1 cells²²⁴. Perfusion-decellularization of pancreatic ECM and its subsequent re-cellularization with β -cells and acinar cells showed upregulation of insulin gene expression in a research²²⁵. Porcine pancreatic dECM has also been used as a scaffold for re-engineering with porcine islets with stem cells, with preserved cell functionality²²⁶. A bioink was developed recently with gelatin and alginate combined with porcine pancreatic dECM and curcumin to produce 3D-printed islet-like structures composed of MIN-6 cells for T1D therapy and protection against inflammatory factors²²⁷.

The encapsulation techniques demonstrated in this chapter show a technique for reproducible production of high concentrations (up to 20 million/mL) of β -cells or islet cells in high-throughput without affecting their functionality, proliferation, or morphology. The developed techniques were further used to generate the core of the artificial islets in the final chapter of this thesis (Chapter 7).

Chapter 7 : Artificial Pancreatic Islets Engineering and Its Potential in Personalized Therapy

Regenerative medicine through cell and tissue engineering has reached worldwide popularity over the recent years. Such therapies have shown great promise for seemingly incurable diseases. Tissue engineering has been explored to engineer organs following a bottom-up approach for restoring the functionality of organs or repairing their damaged structure. In T1D patients, such artificial organoids could help glucose recovery by rebuilding the pancreatic islets, which are destroyed by autoantibodies.

The Pancreatic Islet – Structure and Function

The human pancreas is the only organ in the human body that can perform both endocrine and exocrine functions in the human body. This means that it can modulate the secretion of fluids such as digestive enzymes and blood through ducts and can also secrete hormones which are directly secreted into the bloodstream (**Figure 7-1**). The islets of Langerhans in the pancreas are surrounded by the acinar cells for exocrine functions in a flower-like arrangement. The pancreatic islets have the endocrine cells arranged in a cluster. The majority of these cells are beta cells, which secrete insulin which can break down glucose to the absorbable form, glycogen. Other than insulin, C-peptide and gamma-aminobutyric acid (GABA) are produced by the β -cells. These are produced as by-products of insulin secretion and for the downregulation of glucagon production from the α -cells. The second most common cell type is the alpha cells, which secrete the hormone, glucagon. This hormone acts exactly opposite to insulin and converts glycogen back to glucose. Insulin and glucagon regulate the blood glucose concentration using a feedback mechanism. The other types of cells in these islets include delta cells, F-cells, and capillaries for blood supply. The delta cells secrete the inhibitory hormone somatostatin which has been associated with the inhibition of the growth hormone, and functioning of the gastrointestinal (GI) tract, and also prevents the over-proliferation of certain cells. The F-cells produce pancreatic polypeptides which play a role in digestion and prevent gastric emptying. However, these polypeptides have also been associated with the modulation of insulin secretion to maintain glucose metabolism. Lastly, the endothelial cells are present to form the blood vessels.

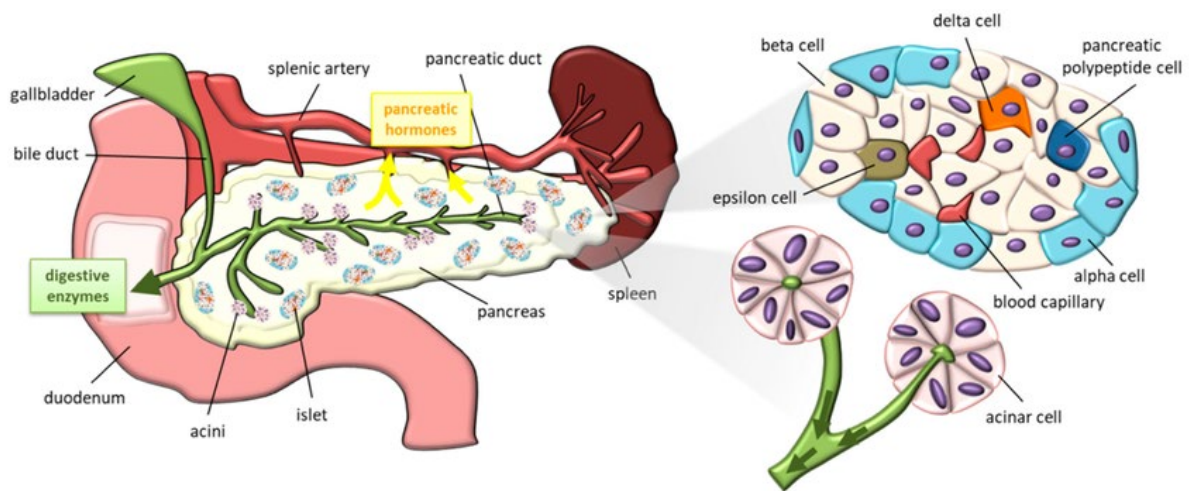


Figure 7-1: Pancreas and its endocrine and exocrine functional parts.

Role of Insulin and its mechanism of action

Insulin is the hormone secreted by β -cells of the pancreatic islets of Langerhans mainly for glucose metabolism. Biochemically, it is a polypeptide consisting of two chains (A and B containing 21 and 30 amino acid residues, respectively) connected by two disulfide bridges with a third disulfide bridge connecting two residues within chain A²²⁸. The insulin receptor is a glycoprotein with two α chains (insulin-binding) and two β subunits (tyrosine kinase and autophosphorylation site). Phosphorylation of the receptor activates insulin receptor substrates (IRS), with IRS1 involved in cell growth and IRS2 in metabolic effects²²⁹. Activation of IRS1 triggers a pathway ending in MAP kinase activation, stimulating cell growth and DNA synthesis. IRS2 activates proteins like PI-3 kinase, linking the insulin receptor to protein 70 kinase, influencing glucose transporter translocation, enzyme activity, and metabolic gene regulation. The insulin has a very short half-life of 3-5 minutes in plasma, after which it is metabolized in the liver and kidneys by insulin-specific proteases for proteolytic cleaving of the molecule or through cleavage of the disulphide bridges by hepatic glutathione-insulin transhydrogenase.

Even though insulin serves a major role in glucose conversion to glycogen, it has several other functions as follows:

- It promotes the enzyme synthesis in the liver for stimulating glycogen synthesis
- It also prevents carbohydrate metabolism processes such as gluconeogenesis (generation of glucose from non-carbohydrate sources) and glycogenolysis (breaking down of glycogen to glucose) by downregulating the enzymes by transcriptional regulation of the hepatic genes responsible for such processes, thereby allowing storage of amino acids and proteins in the cells/tissues²³⁰.
- It promotes absorption of glucose into muscles and cells, which are normally impermeable to glucose
- It facilitates triglyceride storage by forcing cells and muscles to utilize glucose instead of fats

- It promotes lipogenesis in adipose tissues and other cells while inhibiting lipolysis by preventing fatty acid production from these structures for the breakdown of lipids.
- Together with growth hormones, it promotes cell growth by active transport of amino acids into the cells and prevents catabolism of protein molecules.
- It regulates protein synthesis by ribosomes by increasing its cellular content and activating components of the translational machinery (eukaryotic initiation factors (eIFs) and elongation factors (eEFs))²³¹
- It helps in maintaining the $\text{Na}^+ - \text{K}^+$ balance in the body by triggering K^+ to enter muscle cells by increasing the activity of $\text{Na}^+ - \text{K}^+$ ATPase in the cell membrane.

Artificial Organs and Tissue Engineering

Constant improvements in drugs and therapies have revolutionized the field of Medical sciences. Biomedical engineering has emerged as a subfield to integrate engineering into medicine for huge improvements in medicine and treatments. Tissue engineering has gained popularity in recent times for repairing or replacing damaged/diseased tissues or organs while preserving their functions or even enhancing them. The first engineered tissue to be used in clinical practice was skin in 1981, even before the coining of the term “tissue engineering” in 1987²³². The three most important aspects of tissue engineering are: (i) Cells that can proliferate to generate tissues, (ii) scaffolds for anchorage of cells and providing 3D structure for building of the tissues, and (iii) a suitable microenvironment with the correct nutrients, growth factors, gaseous composition, and ambience for growth and functioning of the required tissues.

Stem cells: Basics and their role in regeneration

Stem cells are cells in an undifferentiated form within the different organ systems that can be directed towards evolving (differentiation) into various cell types for repair or regeneration²³³. The capability of a stem cell to be differentiated into a particular cell type is termed potency. The only totipotent or omnipotent stem cells are found at a stage when a zygote is formed, as it can differentiate into any cell type found in the body, and even results in embryogenesis and its development. The embryonic stem cells are the next most powerful cell type in terms of potency, as they can form the three germinal layers (endoderm, mesoderm, and ectoderm), which form the basis of all different functional cells and tissues in the body. Such stem cells are called pluripotent cells. Next in line are the adult stem cells, such as mesenchymal stem cells, which are multipotent. These types of stem cells are present in certain organ systems and have the capability to differentiate into different cell types of the organ system and its related types. Some oligopotent or bipotent stem cells are also present in particular organs for repair and regeneration in the specific organs (for example, lymphoid progenitor cells for immune cell generation, osteoblasts for bone tissue generation). Finally, the specialized unipotent stem cells are present in each organ to differentiate into specific functional cells only for that organ. Due to high-quality research in this field, currently, any type of adult stem cells can be converted to pluripotent cells by induced pluripotency (iPSC). Due to such developments, the stem cells can be directed to differentiate into the desired cell types by providing the required growth factors, thereby altering their genetic expression into that present in the desired cell lineage. This ability has resulted in a boom for using stem cells

in regenerative medicine and tissue engineering to fabricate artificial organs with a specific focus on personalized therapy.

Evolution of the idea of the Project

The initial idea of the project (in collaboration with FBiRN) was to obtain pancreatic islets from a deceased donor, digest the islet-matrix, and extract the islet cells (**Figure 7-2**). Following extraction, the β -cells, α -cells, and endothelial cells from the islets were dissociated using flow cytometry to obtain the separate population and add them to different layers of the core-shell structure obtained directly in a microfluidic FFD-type device using hydrogels to form the artificial islets by crosslinking at 405 nm light.

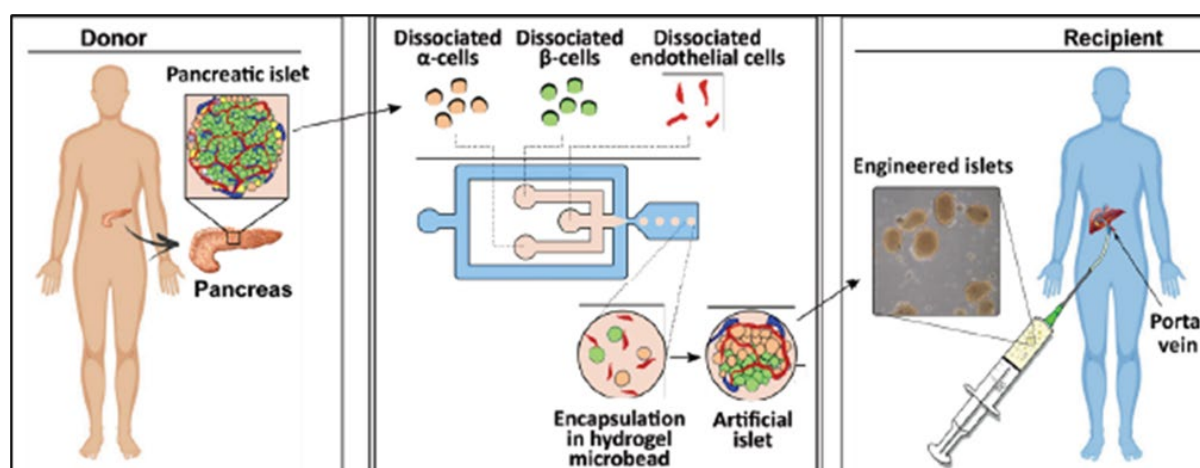


Figure 7-2: The initial idea for artificial islet development.

The final experimental design was modified so as to obtain the best results for viability and functionality of the cells with alternative cell choices to develop the system. However, the basic concept of designing injectable artificial islets in hydrogel droplets was kept unchanged. Based on optimizations during the engineering process, the crosslinking light was changed to 385 nm, and hydrogel compositions were optimized. The core-shell droplet technology was altered to develop an oil-free, soft hydrogel system through simple and rapid photo-crosslinking. Finally, a total human islet cell population or differentiated MSCs were selected to form the core part of the artificial islets to maximize functionality while the process was independent of donor involvement. The modifications improved the system's efficiency while adding a "personalized medicine" approach without compromising the design in any manner.

The final chapter of this thesis deals with the most important part of the project, engineering artificial pancreatic islets. Firstly, the RIN-m or human islet cells in encapsulation were re-encapsulated with HUVECs and tested for glucose susceptibility. To explore our system's application in personalized therapy, we differentiated bone marrow-derived mesenchymal stem cells (BM-MSCs) in ECM-containing GelMA droplets. The finally obtained differentiated cells were found to co-express both insulin and glucagon on immunocytochemistry staining and dithizone staining (selective for insulin). ELISA tests for insulin production were also conducted to prove the efficiency of the systems. Vasculature development of HUVECs in fibrin scaffolds was optimized with the encapsulated pancreatic islet cells and differentiated cells. According to the results obtained, it can therefore be claimed

that artificial pancreatic islets could be developed using microfluidic techniques and can be a promising therapy for type-1 diabetes.

1 Materials and Methods

1.1 Staining of cells with cell tracking dye and re-encapsulation in droplets

For ease of observation and identification of the different cell types in the “cores” and “shell”, the RIN-m or human pancreatic cells (or cells in the “cores”) were stained with green cell tracking dye (CMFDA). Briefly, a 10 mM stock solution was prepared in DMSO, and a 1:500 working solution in serum-free media was made and added to the culture dish with the cells. The cells were incubated for 45 minutes at 37°C and then washed twice with PBS before adding trypsin and detaching the cells for the experiments. After detachment, cells were mixed with the GelMA-HAMA-ECM (GHE) hydrogel pre-polymer at 20 million/mL.

1.2 Insulin analysis and glucose susceptibility test

A 100 μ L of media was collected (and replaced) from each of the differentiated stem cells’ (also controls) wells after differentiation for general insulin production analysis. For the Static Glucose-stimulated Insulin production (GSIS) test, three sets were created with (i) no glucose, (ii) 1 mM glucose (low glucose), and (iii) 15 mM glucose (high glucose). The media collected from the samples were tested according to the protocol provided in the human insulin ELISA kit (Merck GmbH, Germany). Briefly, the known concentration standards and samples diluted with assay buffer were added to each of the wells of the 96-well plate provided in the kit and incubated for an hour. Post-incubation, the wells were thoroughly washed to remove unreacted products/antibodies, and the detection antibody was added. The washing step was repeated, and enzyme and substrate solutions were sequentially added and incubated in a similar manner. After incubation and development of the blue colour after substrate addition, the stop solution was added, and the absorbance was measured at 490 nm using the HTX synergy plate reader. Based on the standard curve derived from the absorbance of the known standards, a graph was plotted to determine the insulin concentrations of the unknown samples.

1.3 Stem cell culture and differentiation experiments

The BM-MSCs were grown in low-glucose DMEM in T25 flasks. They were trypsinized at passage 5 and taken in a 1 million/mL concentration. Different differentiation strategies were tried. Firstly, the 3-step protocol described by **Sun *et al.*** was used for 2D differentiation²³⁴. Briefly, serum-free DMEM high glucose with 0.5 mM/L beta-mercaptoethanol was added for step 1 for 2 days. In the second step, again serum-free high-glucose DMEM was used with 2% B-27 supplement, 1% non-essential amino acids (NEAA, Gibco BRL, Life Technologies, UK), 20 ng/ml β -fibroblast growth factor (bFGF, Sigma-Aldrich, Saint Louis, Missouri, USA), 20 ng/ml EGF(Sigma-Aldrich, Saint Louis, Missouri, USA) for 8 days. In the last step, the cells were cultured in the same DMEM media with different supplements namely 2% B-27 supplement, 10 ng/ml β -cellulin (Sigma-Aldrich, Saint

Louis, Missouri, USA), 10 ng/ml activin A (Sigma-Aldrich, Saint Louis, Missouri, USA), 10 mM/L nicotinamide (Sigma-Aldrich, Saint Louis, Missouri, USA).

To check 3D BM-MSC differentiation in droplets, GelMA-HAMA-ECM hydrogels were used with different ECM percentages (0.5%, 1% and 1.5%). The droplets were grown in high-glucose DMEM for 7 days. A 2D system was tried by adding a layer of the GelMA-HAMA- 0.5% ECM on the well surface and crosslinking for 15 seconds using 385 nm light. Cells were seeded on this layer, and DMEM high-glucose media was added and cultured for 7 days. In both cases, the media was changed to RPMI-1640 (after 7 days) for further culture.

A control sample was kept where BM-MSCs were left to grow in low-glucose DMEM with serum for two weeks.

1.4 Dithizone staining

To test for insulin production from the differentiated BM-MSCs, diphenylthiocarbazone or dithizone (DTZ) staining was done. Briefly, 50 mg of dithizone powder was dissolved in 5 mL of DMSO. This was diluted in a 1:100 ratio in the cell culture media. The cells were incubated in the stain-containing media for 30 minutes at 37°C. The cells were washed with PBS three times and observed under a stereomicroscope to locate cells with red stains.

1.5 Immunocytochemistry staining

Droplets with cells or just the cells were washed twice with cold PBS, followed by the addition of 4% paraformaldehyde (PFA) for fixing the cells. The fixation was done for 1 hour, and then PFA was removed. The cells/droplets were washed thrice with PBS. A 0.1% Triton-X with 2% BSA solution was used for 15-20 minutes for permeabilizing the cells. The cells/droplets were again washed thrice with PBS after incubation. The fluorescence-conjugated mouse monoclonal insulin and glucagon primary antibodies (Invitrogen, Thermofisher GmbH, Germany) were diluted in a 1:10 ratio in blocking buffer (5% BSA with 0.01% Triton-X) and kept at 4°C overnight for antibody binding. The following day the immunostaining solution was washed twice with PBS, followed by the addition of nuclear stain DAPI (2 mg/ml) and incubating at room temperature for 20 minutes. Finally, the wells were washed with PBS twice and air-dried before observing under a confocal microscope at 405 nm for DAPI, 488 nm for insulin, and 570 nm for glucagon.

1.6 Endothelial cell culture and Fibrin hydrogel preparation

A RFP (red fluorescent protein)-modified human umbilical cord vascular endothelial cell (HUVEC) line was used to develop vasculature for the artificial islets. The RFP-HUVECs were cultured in EBM (endothelial basal medium) with supplements (Lonza, Germany). The cells were used between passages 3-5 for experiments after trypsinization.

The preferred hydrogel for HUVECs for growth, proliferation, and vasculogenesis is fibrin. To produce this hydrogel, 2.5 mg/mL of fibrinogen (Sigma-Aldrich, Germany) was dissolved in PBS. To this solution, a 1:3 diluted aliquot of the enzyme, thrombin, was added. After 15 minutes of incubation at 37°C, the fibrinogen was converted into the insoluble form,

fibrin. For droplets, the fibrinogen solution was mixed with the photocrosslinkable hydrogel pre-polymer and converted into HUVEC-laden droplets using the re-encapsulation chip. After crosslinking over 385 nm light, the droplets were washed with PFO and Novec and then replaced with the thrombin solution instead of PBS and incubated as described earlier. Post-incubation, the thrombin was removed, and the droplets were washed with PBS twice before adding cell culture media.

1.7 Image acquisition and data analysis

Images were acquired using the Nikon confocal A1 microscope or the uEye camera (iDS, Germany) and software. Images were analysed using the ImageJ software or the Nikon A1 analysis software. Graphpad Prism was used for graphical analysis.

2 Results and Inferences

2.1 Stem-cell differentiation to pancreatic islet cells

2.1.1 Optimization of the differentiation method

Different differentiation protocols were attempted to obtain pancreatic islet-like cells from BM-MSCs. Initially, during the passaging of BM-MSCs, it was noted that culturing in a T25 flask instead of a T-75 flask helped in faster proliferation of BM-MSCs. For differentiation, cells at passage 6 were used. Among the 2D differentiation methods, the following methods were tested:

- The 3-step differentiation protocol (described by Sun *et al.* described in the Materials and methods section).
- DMEM - high glucose media was initially added for 7 days, followed by the addition of RPMI-1640 media.
- GelMA-HAMA- 0.5% ECM was coated on the surface of the culture dish and crosslinked. The dMSCs were cultured on this.

The cells cultured in DMEM low glucose in 2D were considered the control cells. Firstly, it was observed that by day 2, the 3-step differentiation cells already started showing an altered morphology after the addition of β -mercaptoethanol with high-glucose DMEM (**Figure 7-3**). For the second case, the morphology of the cells did not show any noticeable changes compared to the control sample. For the third case, a few circular cells started appearing on day 2 of culture.

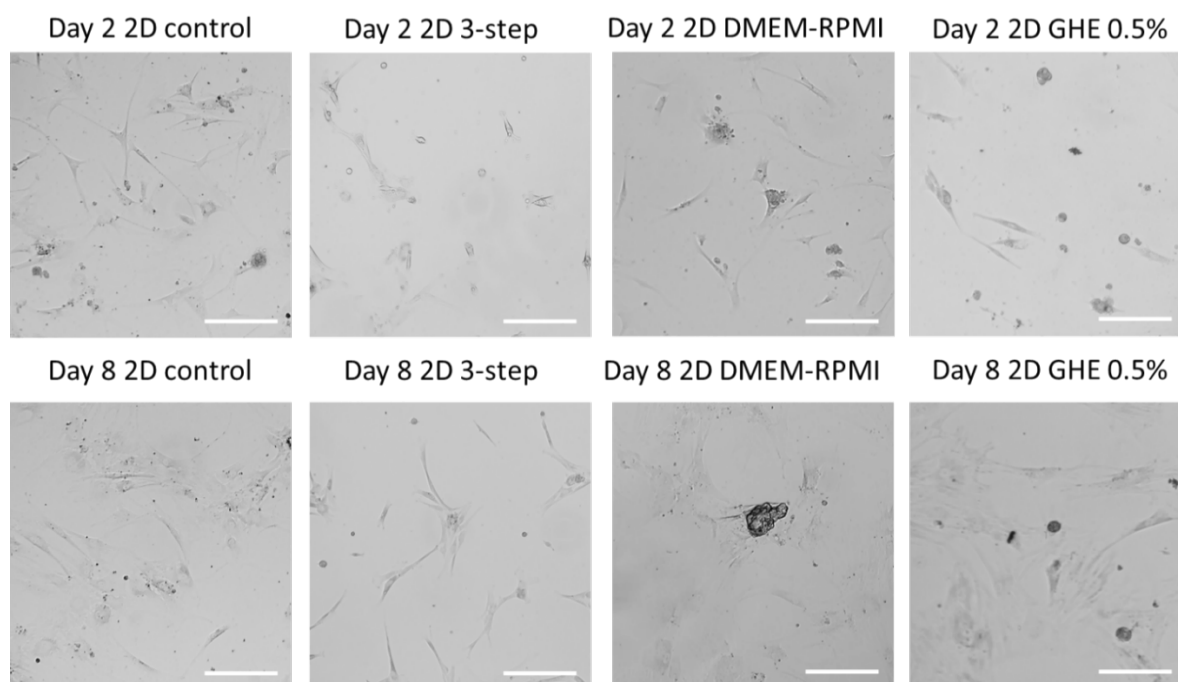


Figure 7-3: Differentiation attempts on BM-MSCs in 2D culture (Scale bar = 200 μ m).

After 8 days of culture, tissue-like conjugated cells appeared in the control cells as well as the DMEM-RPMI cells (after addition of RPMI media only). In the RPMI-cultured cells, the appearance of darker granulated structures was also visible. For the 3-step differentiation, the second stage was ongoing, and cells had an elongated morphology and were probably undergoing neurogenic differentiation (as supposed). In the case of the GHE-coated dish, the circular structures became more prominent while cells appeared elongated as well. Tissue-like cell-aggregates were also seen in this case.

In the case of the other set of differentiation protocols in 3D, the 3-step differentiation protocol was also tested in GelMA 4.8% droplets. For the other cases, the concentrations of ECM were varied in the different sets with (i) 0.5%, (ii) 1% and (iii) 1.5% ECM, combined with 6% GelMA and 0.5% HAMA in the initial composition before mixing with cells. The droplets were cultured in DMEM high-glucose medium and then shifted to RPMI after day 7. Generally, in the droplets, the cells were in a rounded morphology throughout in most cases due to the crosslinked hydrogel pores (**Figure 7-4**). It was difficult to figure out whether the cells were undergoing differentiation just by morphological analysis of microscopic images.

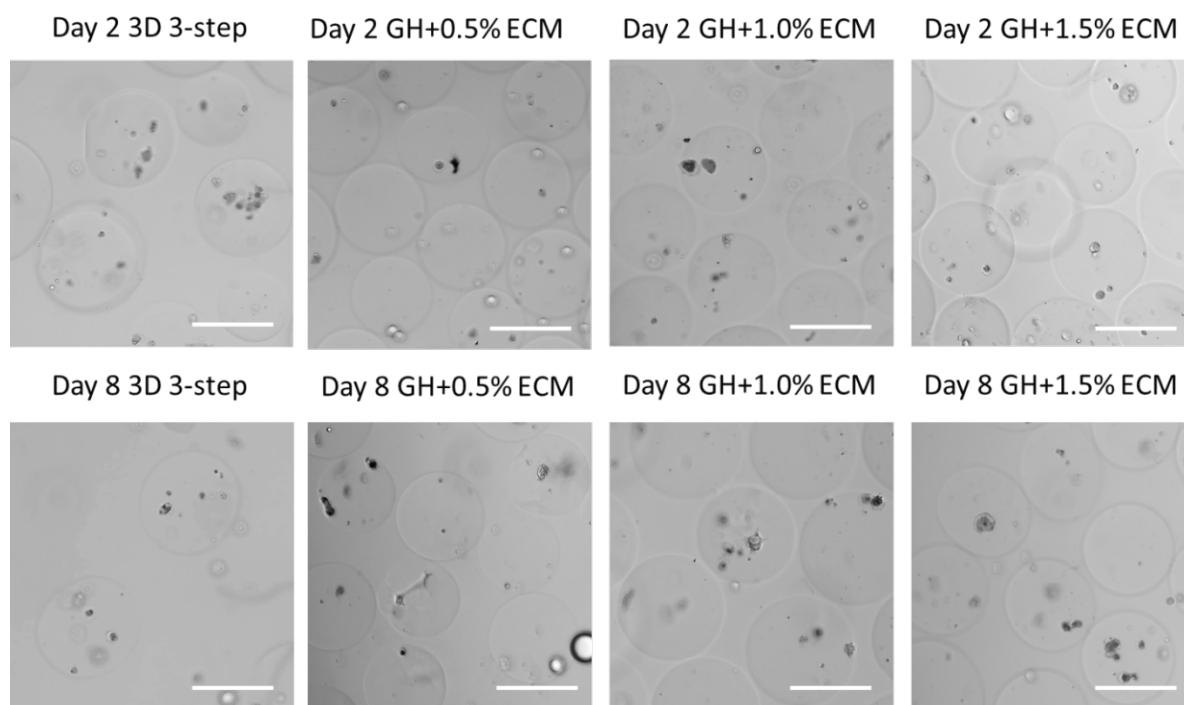


Figure 7-4: Differentiation attempts on BM-MSCs in 3D culture encapsulated in droplets (Scale bar = 200 μ m).

On very careful analysis, it was observed that the MSCs in the GelMA droplets undergoing 3-step differentiation became smaller by day 8 compared to their morphology on day 2. This might be due to encapsulation stress or a result of ongoing differentiation. For the GH with ECM droplets, some of the cells migrated to the droplet surface and attained different morphologies, while the others continued to retain their rounded structure in encapsulation. The encapsulated cells grew darker over time. However, further tests were necessary to confirm whether the cells in the droplets had differentiated or not.

2.1.2 Confirmation of differentiation by dithizone staining

The first confirmatory test done to understand the efficiency of differentiation for the different protocols was dithizone (DTZ, diphenylthiocarbazone) staining. The DTZ acts based on zinc chelation in the zinc-rich pancreatic β -cells (or insulin-producing cells), resulting in a red colouration²³⁵. The zinc ions (Zn^{2+}) bind to the insulin hexamer stored in all insulin-secreting vesicles, which is carried to the site of action by zinc-transporters. The test was performed on all the differentiated cells after day 18 of culture (especially for the completion of the 3-step protocol). Among the 2D differentiation protocols, cells grown on the GHE-coated surface and in the DMEM-RPMI media both showed the presence of some red, DTZ-stained cells (**Figure 7-5**). However, the 3-step differentiated cells, both in 2D and 3D, did not show the presence of DTZ-stained cells, indicating that possibly the differentiation method did not work or had very low efficiency.

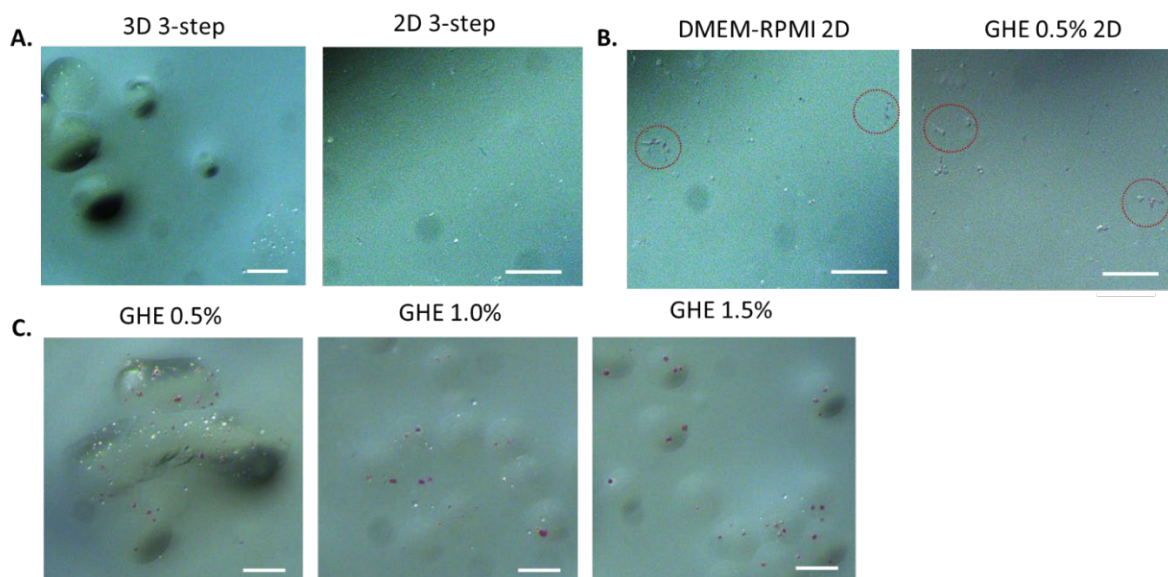


Figure 7-5: Dithizone staining of insulin-positive cells after BM-MSC differentiation. **A.** Cells after 3-step differentiation, **B.** Cells showing DTZ staining (in red circles) after 2D differentiation protocols, and **C.** Cells differentiated in droplets showing more DTZ-stained cells for higher ECM concentrations in droplets.

The 3D cultured cells in different ECM concentrations showed the presence of red-stained cells in the droplets in all three cases. The percentage of red-stained cells was directly proportional to the amount of ECM present in the hydrogel composition. The clear dots in the droplets indicated the unstained cells. It appears that the pancreatic dECM provided the necessary cues for differentiation of the BM-MSCs. More confirmatory tests were performed to check if the 3-step differentiation did not result in any differentiation at all and also to confirm the results for the other samples.

2.1.3 Immunocytochemistry for functional analysis of differentiated cells

Following the dithizone staining, immunochemistry was done using mouse monoclonal insulin (Alexa Fluor™ 488, eBioscience™) and glucagon (eFluor™ 570, eBioscience™) antibodies and DAPI as a counterstain (for nuclei). The ICC was also done after day 18 of culture, after fixing the cells. Even though extremely reduced in intensity and area, slight binding of insulin antibodies was observed in the control cells along with a slightly wider coverage of the glucagon antibodies (**Figure 7-6**). This may be due to the low-glucose media used for maintaining these cells. For the 2D 3-step sample, the area of insulin and glucagon detection was even smaller, even though the intensity was increased (for insulin). This may also be due to the orientation of the cell, and cannot be used as a comparative factor.

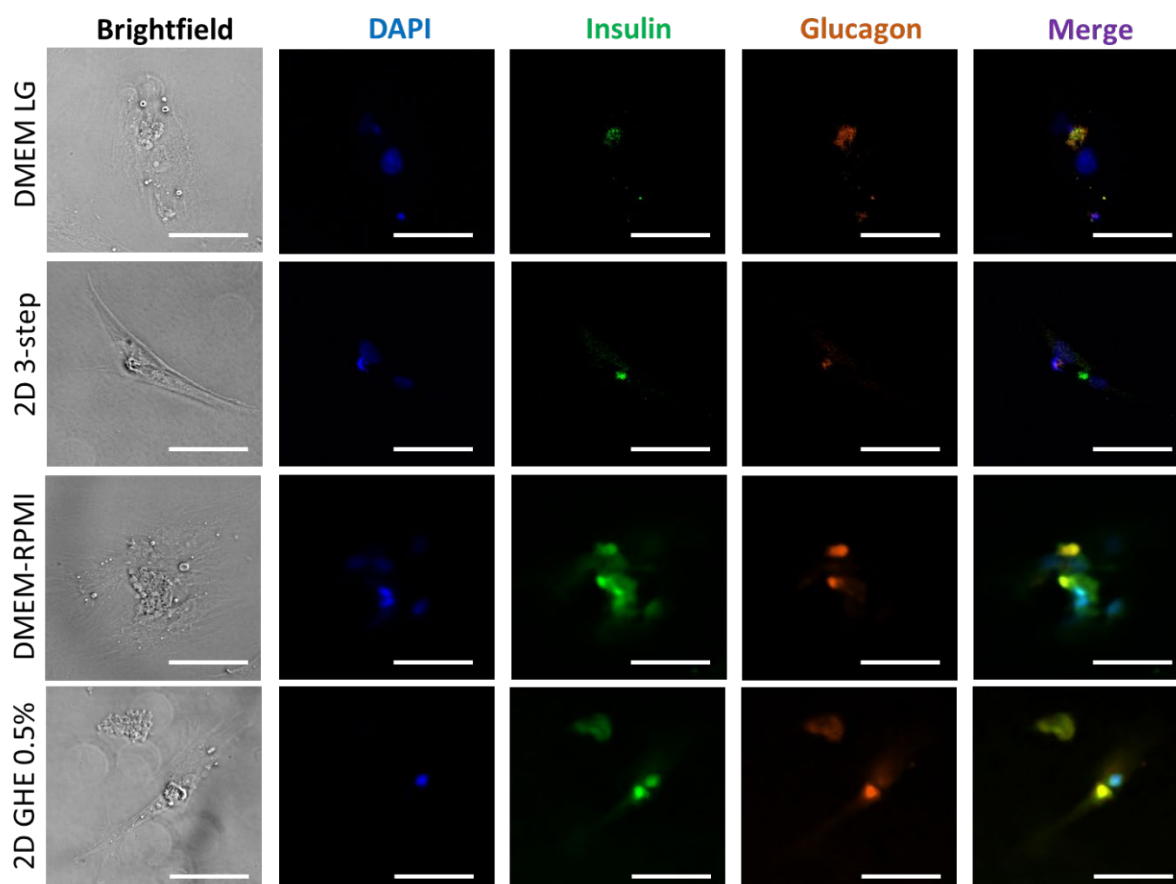


Figure 7-6: Immunocytochemistry staining of the 2D differentiated cells with insulin (green) and glucagon (orange) antibodies. The cells were counterstained with DAPI (blue) for the nucleus (Scale bar = 10 μ m).

The DMEM-RPMI cells and the cells grown on GHE coating both showed a significant area of insulin expression, with the latter showing most of the detected areas co-expressing both insulin and glucagon. This correlates with the results obtained from the DTZ staining.

For the 3D droplet differentiation samples, the droplets were dissolved by treating with 1 mg/mL collagenase for 3 minutes in the incubator at 37°C. After dissolution of the droplets, the enzyme was neutralized using FBS-containing media. The cells were placed in culture dishes and allowed to attach overnight. Post-attachment and the ICC staining protocol, the 3-step differentiation sample was again found to have a low fluorescence intensity of both insulin and glucagon (**Figure 7-7**). However, the cells showed co-expression of insulin and glucagon.

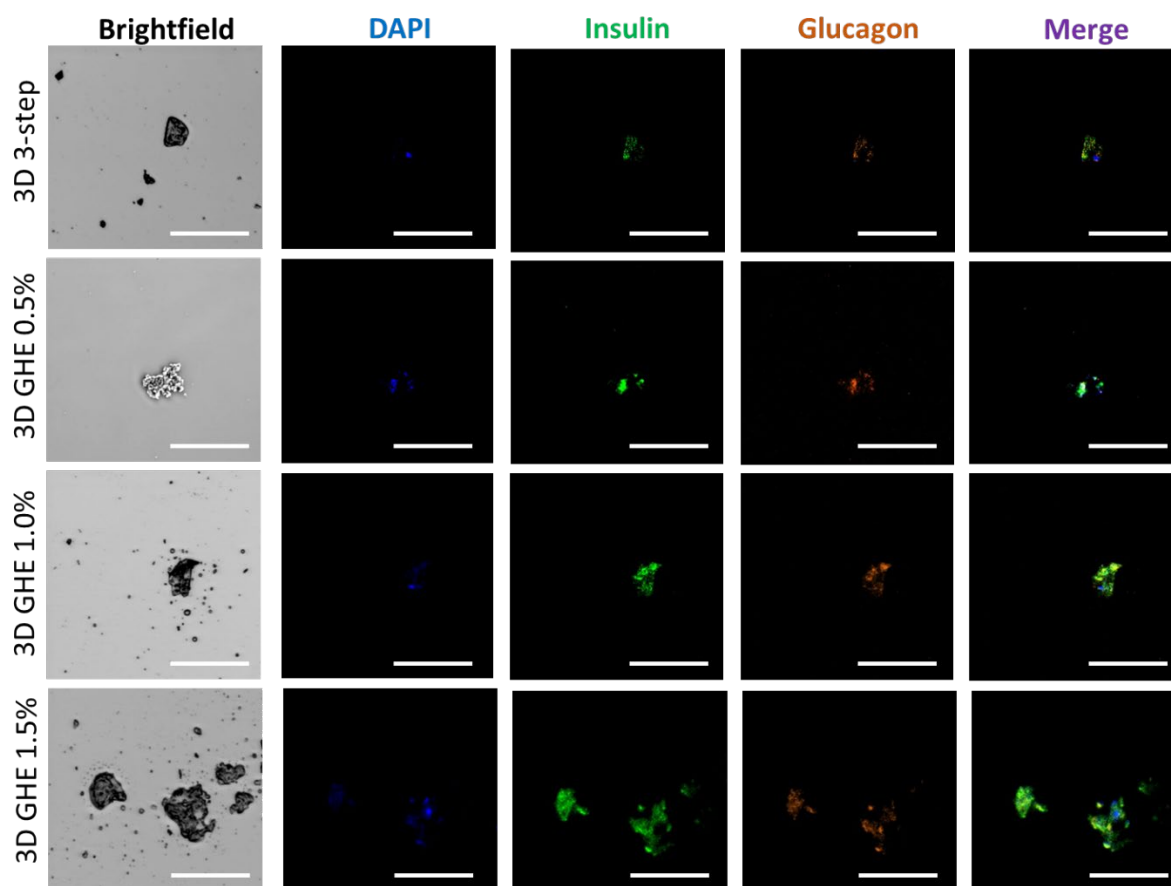


Figure 7-7: Immunocytochemistry staining of the cells attached to the culture dish after extracting from the droplets after Day 18 of culture with insulin (green) and glucagon (orange) antibodies. The nuclei are counterstained with DAPI (Scale bar = 10 μ m).

For the cells differentiated in ECM-containing droplets, co-expression of insulin and glucagon was observed in all samples. The fluorescence area for the GHE 0.5% sample was also quite small, but the fluorescence intensities were strong. The sample with the highest amount of dECM showed the highest area of insulin/glucagon coverage. This might be due to the slightly larger cells that were observed in the case of the GHE 1.5% sample, coincidentally. Hence, it can be assumed that the DTZ staining results were overall fairly relatable to the ICC results.

2.2 Re-encapsulation attempts with endothelial cells and Vasculature optimization

2.2.1 Re-encapsulation of RIN-m and human islet cells with HUVEC

The first tests for attempting to develop the vasculature for the islets were done by using RIN-m cells in the core. The core hydrogel was kept constant for all tests based on the optimizations in Chapter 6. The RFP-HUVECs (red) were used for the development of vasculature in the shell of the artificial islets. Fibrin (2.5 mg/mL of fibrinogen in the initial concentration) was used as a major component of the shell to enable growth factors to be released for stimulating vasculogenesis²³⁶. GelMA (6% initial concentration) was added to add durability to the shell with the fibrin. During this test, RIN-m cells showed formation of spheroids for the first time (**Figure 7-8.A.**) after a week in culture. This might be due to the presence of the HUVECs in close vicinity or the presence of fibrin in the shell, or a combined

effect of both. The spheroids, however, had a tumour-like characteristic and could penetrate through both the core and the shell. For the HUVECs, however, the GelMA seemed to affect their vasculogenesis and proliferation rates, with just a few proliferated cells even after 18 days of culture.

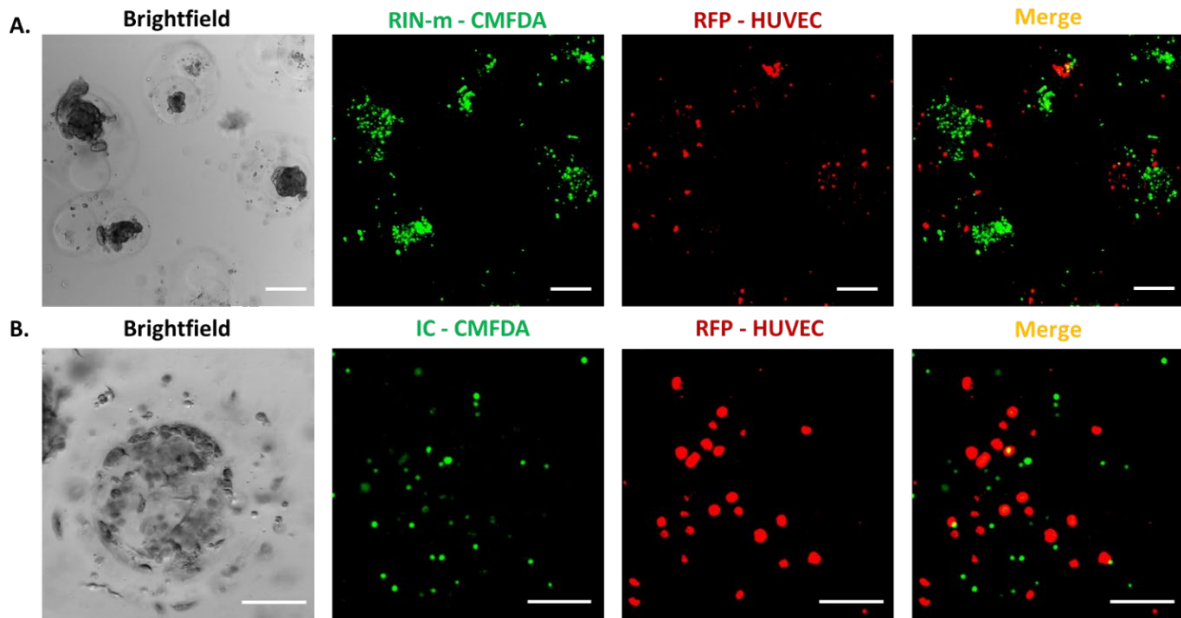


Figure 7-8: Re-encapsulation of HUVECs in the shell for vasculature development. **A.** RIN-m cells encapsulated in GHE core with RFP-HUVECs in GelMA-fibrin cells on day 18 of culture (Scale bar = 200 μ m). **B.** Human ICs in GHE core with RFP-HUVECs in HAMA-fibrin shell on day 21 of culture (Scale bar = 100 μ m).

Another hydrogel composition for the shell was tried to maintain the structure without significantly affecting the cells. A 1% HAMA was added with fibrin instead of GelMA to decrease the hydrogel content of the shell. The cores were used to encapsulate ICs instead of the insulinoma cells. The ICs did not form any spheroids even after 3 weeks of culture, indicating that the feature might be restricted to the RIN-m cells due to their tumourigenicity (**Figure 7-8.B.**). However, clustering of cells and even tissue formation on the cores from the islet fibroblasts was visible. The HAMA-fibrin shell also did not show signs of any vasculogenesis, even on day 21 of culture, and needed further optimizations.

2.2.2 Testing of functionality in re-encapsulated islet cells and differentiated stem cells

The differentiated cells and the re-encapsulated ICs were checked for functionality based on insulin production capabilities. Firstly, 50 μ L of the culture media was stored after 1 day of culture with the so-called differentiated cells (DMEM high glucose from the 3-step differentiated cells, DMEM low glucose from the control, and RPMI from the rest of the samples). This media was used to test the insulin production using the ELISA assay. As was observed from the ICC and DTZ staining tests, the 2D differentiation using the 3-step protocol did not work, and hence no insulin secretion was observed from this sample. The 3D version of the same showed a very small amount of insulin production, but it was indicative of a low differentiation efficiency. Among the 2D differentiated samples, the RPMI-grown samples showed the best insulin production levels (~ 45 μ U/mL). Among GHE samples, the 2D version

with 0.5% ECM showed better insulin production compared to the 3D version (~15 $\mu\text{U/mL}$ higher secretion). This might be due to the space available to acquire the preferred morphology by the cells in 2D. However, the best among all samples was found to be the GHE with 1.5% ECM for 3D differentiation with levels of up to 60 $\mu\text{U/mL}$. These results also correlated with the DTZ staining results. It can hence be confirmed that the dECM and RPMI-1640 media can both play an important role in the differentiation of the BM-MSCs to produce functional pancreatic islet-like cells.

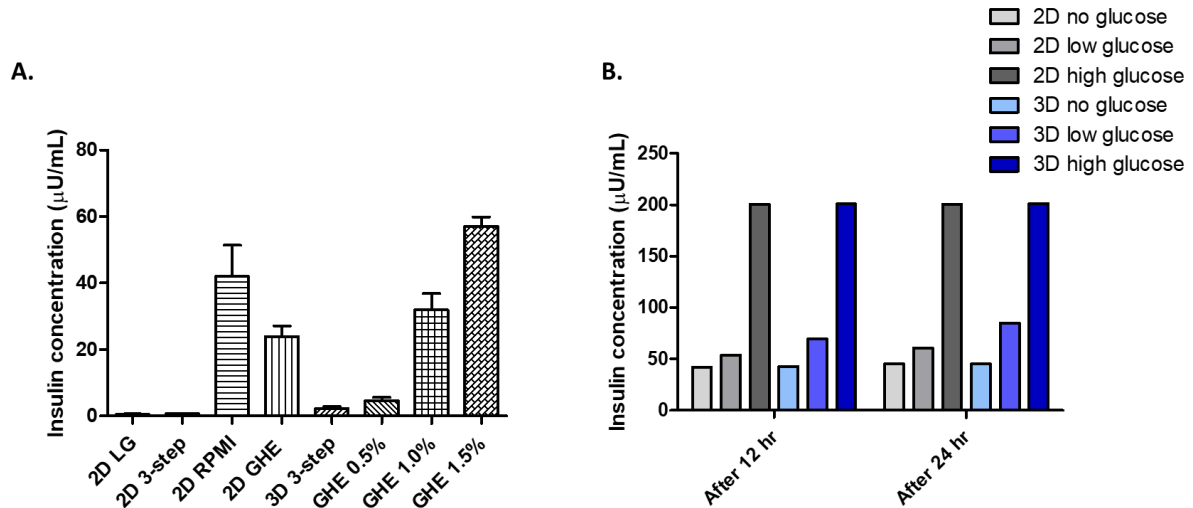


Figure 7-9: Insulin production levels detected by ELISA assay. **A.** Insulin levels produced from the different differentiation approaches for converting BM-MSCs to pancreatic islet-like cells. **B.** GSIS test performed for re-encapsulated ICs with RFP-HUVECs using no, low (1 mM) or high (15 mM) concentrations of glucose in the culture media over 24 hours of culture.

A glucose susceptibility test was performed for the re-encapsulated ICs with RFP-HUVECs over a one-day period and compared to 2D culture. Three different conditions were considered with (i) no glucose added to the culture media, (ii) 1 mM (low concentration) glucose, and (iii) 15 mM glucose added to the culture media. The test was performed after two weeks of culturing the cells in their respective conditions. The insulin secretion ranges were comparable for both the 2D and re-encapsulated (3D) cells in most of the cases (**Figure 7-9.B**). Also, most of the insulin secretion happens within the 12 hours of the glucose addition, which slightly increases over the next 12 hours. For the low glucose cases, the 3D cells outperformed the cells in 2D culture. This proves that the multiple hydrogel layers do not limit insulin production from the cells and can even add to the better functionality of the cells.

2.2.3 Fibrin hydrogel crosslinking optimization for vasculature development

Even though the re-encapsulated ICs with RFP-HUVECs in Fibrin-HAMA shells allowed good functionality to the pancreatic islet cells, the HUVECs did not reach their full potential of forming vasculature to form the desired artificial islet structure. To sort the issue, firstly, the fibrin crosslinking with thrombin was optimized based on the fibrinogen to thrombin volume ratio. 25 μL of fibrinogen (2.5 mg/mL of media) with 5 million HUVECs per mL was mixed with empty GelMA-HAMA-ECM droplets to mimic the islet structure and was added to 4 wells in a 96-well plate. To each of the wells, different thrombin (1:3 diluted in PBS) were added (25, 50, 75, and 100 μL , respectively). During the mixing process for the highest volume

ratio of 1:4 of fibrinogen to thrombin, the mixing was difficult as aggregation/crosslinking started sooner. For the rest of the conditions, this problem was not observed.

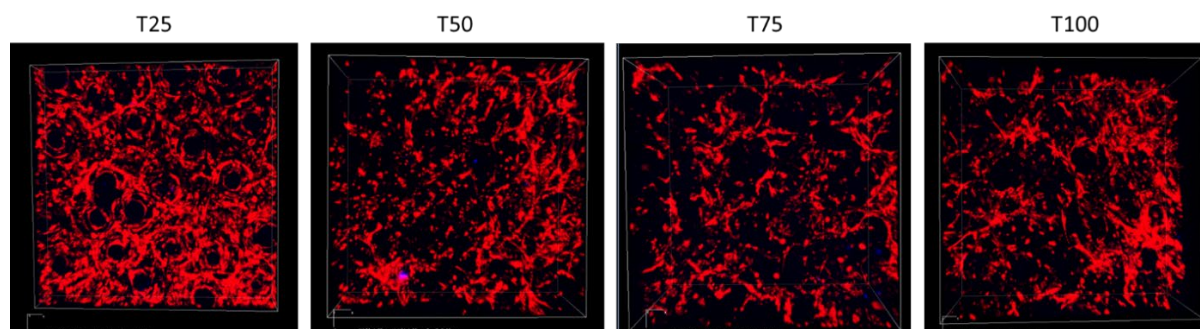


Figure 7-10: Thrombin (T) concentration optimization to obtain the desired fibrin structure for vasculature development (3D view).

After 14 days of culture, proper vasculature formation by the HUVECs was observed surrounding the GHE droplets. The vascular connections were the best for the 1:1 fibrinogen to thrombin ratio, indicating that the softer hydrogels were better for the vascularization (**Figure 7-10**). In cases of higher thrombin concentrations, the parts with stronger crosslinking showed worse connectivity between the HUVECs, and parts where the hydrogel density was lower (near the edges of the wells), the connections were much more prominent.

2.3 Artificial islets generation

2.3.1 Integrating HUVECs and fibroblasts into the system

Before shifting to the re-encapsulated artificial islets structure, an alternate system was attempted. Since the GHE droplets in fibrin were quite stable, and great vasculature formation was observed in the fibrin formed by 1:1 mixing of fibrinogen to thrombin, an islet disc with the same design was tested. ICs labelled with CMFDA were encapsulated in GHE cores and mixed with the fibrinogen just as in the previous Section 2.2.3. Both the HUVECs and ICs proliferated well in the setup; however, the vasculature formation was slower than the rates observed with empty droplets (**Figure 7-11. A.i**). This is probably due to the competitive growth of the ICs simultaneously, which also takes up a part of the nutrients available in the system, limiting the availability to the HUVECs. On closer inspection, it appeared that the ICs were mostly migrating from the droplets (**Figure 7-11.B.**). However, the random distribution of the droplets within the fibrin, with droplets overlapping in several regions in 3D space, made it very difficult to figure out the specific localizations of the cells. Additionally, the discs were quite large (around 0.32 cm^2 , approximately the area covering the well bottom of the 96-well plate). Though this size regime is fine for transplantation purposes but it is unsuitable for injectable therapies. This system helped understand certain important features of the cells during culture, which were utilized for improving the final islet design.

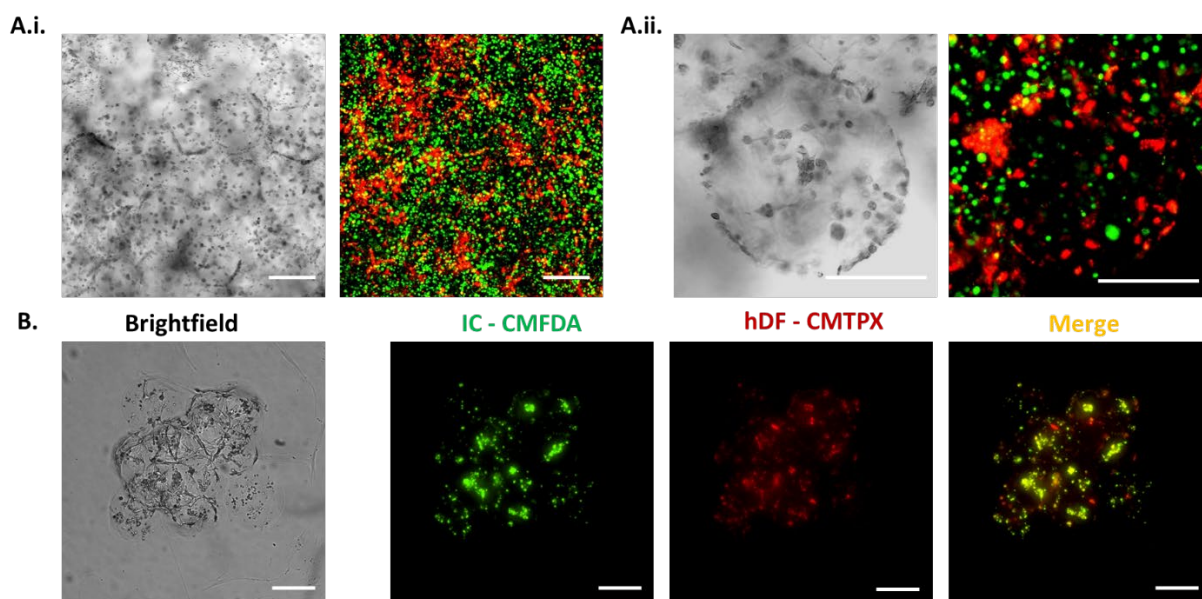


Figure 7-11: Adding RFP-HUVECs and hDFs around the droplets with islet cells. **A.** GHE droplets with CMFDA-labelled ICs (green) cultured in encapsulation with fibrin containing RFP-HUVECs to form islet discs in a 96-well plate **i.** vasculature formation observed surrounding droplets on Day 20 of culture (Scale bar = 200 μ m). **ii.** A single droplet connected to other droplets observed within the fibrin encapsulation at higher optical magnification (Scale bar = 100 μ m). **B.** Attachment of fibroblasts (hDFs; red CMTPIX labelled) to the GHE droplets containing CMFDA labelled ICs (green) after 7 days of culture (Scale bar = 200 μ m).

In another experiment, the idea of including fibroblasts into the system was checked. A 5 million/mL concentration of fibroblasts (hDFs labelled with CMTPIX) was added to GHE droplets with ICs (CMFDA labelled) growing in 48-well plate wells. It was observed that the fibroblasts preferred growing over the droplet surfaces compared to the bottom of the wells (**Figure 7-11.B.**). Their proliferation rates are quite high, and by the end of a week, the fibroblasts form tissue-like structures, binding the droplets together, forming a droplet cluster. This feature can help in better crosstalk between the cells and enhance functionality as the islet cells prefer working in clusters.

2.3.2 Artificial islets in droplets with vasculature

The final artificial islets were designed based on all the optimizations and characteristics of each of the components used in the system. The GelMA 4.8% + HAMA 0.4% and ECM 0.4% final composition was used to encapsulate 20 million/mL of the ICs using the OTF system. Differentiated stem cells were not demonstrated with this system due to low yields and a very slow initial proliferation rate of the BM-MSCs. Following encapsulation, the droplets were mixed with the shell hydrogel pre-polymer. For the final optimization, 4 hydrogel compositions were tested. GelMA 2% with 0.2% HAMA was constant in all these compositions, and the required additions were made to obtain final concentrations of the following: (i) 0.5% of dECM, (ii) 0.5% ECM with 1.25 mg/mL of fibrinogen, (iii) 0.5% ECM with 10 ng/mL VEGF, and (iv) 0.5% ECM with 1.25 mg/mL of fibrinogen and 10 ng/mL VEGF. A 1:1 concentration of HUVECs to fibroblasts was added to the shell pre-polymer in a 10 million/mL concentration.

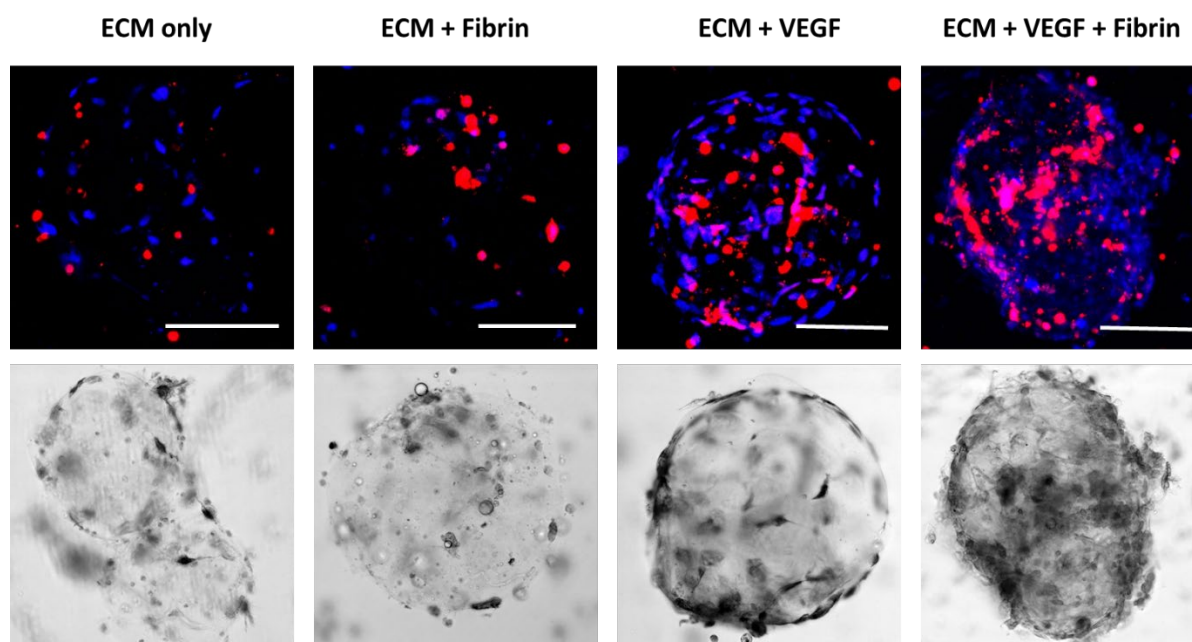


Figure 7-12: Artificial islets developed with islet cells in GHE cores and RFP-HUVECs and hDFs in shells with different hydrogel compositions to sustain vascularization (All cells other than RFP-HUVECs were labelled with Hoechst 33342-blue for nuclei labelling) on Day 21 of culture.

Out of the 4 compositions tested, the artificial islets with ECM and ECM and fibrin only in the shells showed poor cell proliferation even after 20 days of culture (**Figure 7-12**). This is mostly due to the low hydrogel composition of the shell, which leads to dissolution of the shells in most cases, mainly for the ECM-only samples. For the fibrin samples, it is visible that just fibrin is not enough to produce a stable structure for the vasculature development, especially in the presence of other hydrogels in the composition. Using Fibrin only for generating the shells has been attempted during this project, but the structure was not stable in culture beyond 1 day. The addition of VEGF to the system shows a significant improvement in HUVEC proliferation and triggering vasculogenesis after day 10 of culture. The best case was observed with a combination of GelMA, HAMA, ECM, and Fibrin with added VEGF to the pre-polymer. The fibroblasts had a very high proliferation rate and rapidly outgrew all the other cells to form a network of tissues surrounding the re-encapsulated islets. For future tests, reduction of the hDF concentration might lead to faster vasculature development.

The aim of the project was thus fulfilled by generating stable artificial pancreatic islets with the use of microfluidics to generate the organoids. The islet-like structures were checked for effects on viability, functionality, and stability in culture in the long term.

3 Discussion

[Artificial islets: idea behind the formulation]

A strategy for T1D treatment with a bioartificial pancreas was suggested by Chang as early as 1966, where he suggested encapsulating pancreatic islets in hydrogel membranes made of alginate-poly(l-lysine)alginate²³⁷. Though several studies have focused on encapsulation of

pancreatic islets for transplantation purposes, engineering of pancreatic islets is a fairly recent idea. In a study, three cell lines (α , β , and pancreatic epithelial cells) were mixed in a 2:7:1 ratio to obtain spheroids in wells capable of secreting the islet hormones²³⁸. However, no use of microfluidics or hydrogels was done for this system, thereby making the organoids still susceptible to immunogenicity. Among methods with hydrogels, Laporte and colleagues used M-rich alginate (with added RGD motifs) to encapsulate human islets with MSCs to improve the survival and functionality of the islets²³⁹.

[Capabilities of the system]

The artificial islets developed during this project are capable of maintaining their structure and function while maintaining high-throughput of generation and efficient production periods. The use of microfluidics in the generation of these islets enables rapid production rates and good uniformity due to control of the mechanical parameters, as the process is not prone to variations due to human handling. The added advantage for long-term survivability of the islets is due to their micro-size, allowing better exchange of nutrients and gases. Previously, it has been demonstrated that breaking down rat islets into single cells and re-aggregating them into such micro-islets in alginate encapsulation led to better viability and oxygen availability to cells and, in turn, enhanced their function and lowered inflammatory factors post-transplantation²⁴⁰. The composite hydrogel that we have used for the fabrication of our islets (both the cores and the shell) helps provide the required microenvironment to sustain the islet cells and facilitate the development of vasculature due to the presence of the porcine pancreatic dECM and the fibrin-VEGF, respectively. The GelMA and HAMA serve as durable matrix materials for supporting cell growth and function while maintaining the droplet structures for very long periods of time (more than a month in culture). The hydrogels can also provide protection to the encapsulated cells against immune-mediated damage, which is a major problem for T1D. As all the hydrogel components are animal-derived, the generated artificial islets are completely biocompatible.

[Advantages and disadvantages of the stem cell-based system]

The stem cells (BM-MSCs) used in our system are adult stem cells, which can be derived from the T1D patient's body, thereby preventing factors such as immune rejection after transplantation. Since we can directly differentiate the MSCs to pancreatic islet cells within the GHE droplets, the droplets can be used as cores for re-encapsulation directly after differentiation without any added steps, such as enzymatic detachment from culture dishes, causing minimal effect on the cells' state. The differentiation process is very simple and requires no additional expensive growth factors, and can be performed with a commonly used culture medium like RPMI-1640.

The stem cell-based system, however, has certain limitations. Firstly, the yield of cells is pretty low. Based on our cell line-based studies, we used a 20 million/mL concentration of cells for the cores to obtain highly functional islet-like structures, with each core having an average of around 50-100 cells. However, for the stem cell system, reaching this high number of cells is a difficult task. Moreover, such a high cell number in encapsulation can limit the differentiation potential and efficiency of the MSCs. Currently, a maximum of 5 million/mL cell concentration has been attempted in the droplets with good differentiation efficiency. For application-related limitations, obtaining the MSCs from the bone marrow is a painful procedure, even though it might be needed just once. This can be avoided by shifting the focus

from MSCs to iPSCs, which can be generated from any stem cell type obtained from a different source.

[Applications of such artificial organs in different physiological systems]

In clinical practice, several artificial organs/tissues/scaffolds have already been implemented. The first ones to be approved include synthetic or mechanical structures with proven biocompatibility, such as valves/stents for cardiovascular functions, metallic frames for bone regeneration, or dialysis devices for replacing kidney functions. Based on modern tissue engineering techniques, some artificial organs have been developed. For the cardiovascular system, a bioartificial heart has been developed with cardiomyocytes seeded in the decellularized matrix from the whole heart²⁴¹. An MSC-derived cardiomyocyte system with decellularized cardiac tissue scaffolds has been shown to obtain good cardiac functionality²⁴². Several such attempts are being made to improve these structures and implement them²⁴³. Most of the approved tissue-engineered structures have been from the sector of bone/cartilage tissue engineering²⁴⁴. Similarly, such structures have been developed for bioartificial lungs, pancreas, liver, and kidneys²³². The system damage-directed approaches in tissue engineering have led to the development of complex organ systems as well, such as the research for the development of artificial nervous systems to restore functionality as treatment for chronic stroke²⁴⁵. Altogether, such systems have outstanding potential in simplifying and personalizing therapeutics with highly rewarding outcomes in regenerative medicine.

Future Perspectives of the Project

This project for developing cell encapsulation models for cancer and artificial pancreatic islets had a highly multi-disciplinary approach with the perfect amalgamation of the best of several fields, such as microfluidics, biomaterial development, 3D cell culture, tissue engineering, organoid development, therapeutics, and even personalized medicine. Based on the obtained results, it can be claimed that functional artificial islets were obtained, which can be taken to clinical trials as the next step. Specifically, the artificial islets could be delivered to the site of action in the pancreas by injecting them through the hepatic portal vein in mouse models. This will set the course for clinical implementation of these artificial islets for T1D therapy.

The system design can be further improved to make it completely automated. This can be done by modifying the OTF system in a way so as to develop a completely automated encapsulation process to obtain crosslinked cores and directly carry them to the re-encapsulation chip to reduce variabilities due to human handling even more. For the islets, another approach can be tried to replace MSCs with iPSCs to enhance the system's applicability. Also, further functionality tests can be performed on the designed artificial islets, such as tissue sectioning and high-resolution imaging, to find the localization of the cells within the hydrogel. Even time-dependent observation of tissue formation can be investigated to understand the effect of the design parameters better.

As for the different engineered chips, crosslinking methods, droplet production techniques, and hydrogel compositions developed during the course of this project, they can be applied in a multitude of other applications. The robustness of the system allows modification or usage of different hydrogels and use of varied cell types, thereby significantly broadening its applicability to a great extent. Altogether, each of the optimizations and designs developed during this project can open up possibilities of further research in diverse directions.

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