Design, evaluation and use of a microfluidic device for automated production of mucin microparticles for cell encapsulation for diabetes therapy

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Design, evaluation and use of a microfluidic device for automated production of mucin microparticles for cell encapsulation for diabetes therapy.

Swedish Title: Design, utvärdering, automatisering och användning av en mikrofluidisk krets för produktion av mucin mikropartiklar för cellinkapsling för diabetesbehandling

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Abstract

Droplet microfluidics has prevalent growth in a wide range of applications in the life science field. This project is one such example where we used a droplet microfluidics tool for treating diabetes mellitus type 1. Mucin hydrogels (Muc-gels) modulate the immune response driving biomaterial-induced fibrosis.[1]. In this project, we have fabricated a droplet microfluidic device for encapsulation of Min6m9 cells (Insulin secreting cells) in mucin hydrogel microparticles. Initially a novel microfluidic circuit design made for production of droplets and fabricated the same by micromachining and 3D printing. Before producing cell encapsulated mucin hydrogel droplets, we have evaluated the functionality of the device by using water as a model we have tested mixing of components from two inlet fluid channels, the size distribution of water droplets produced at different pressure values, droplet generation rate. Thereafter, Min6m9 cells are encapsulated in mucin microparticles, viability studies have been made and observed growth of cells which eventually forming islet-like structure after 15 days of incubation. Performed experiments to make microfluidic device to work in an automated format by using a fluid handling robot for mass production and solving other problems that occurred while encapsulation.
Sammanfattning

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I would like to thank my supervisor Håkan Jönsson for all support in my work with my master thesis. H.Jönsson have introduced me to the Nanobiotechnology group at SciLifeLab, been very supportive and given me valuable feedback and helped me at each and every step in my thesis work. I really enjoyed having discussion about my project under him. Also I thank him for allowing me to attend lot of PhD defence parties and microfluidic laboratory visit at Uppsala.

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Chapter 1

Introduction

1.1 Droplet Microfluidics and Type 1 Diabetes

Malfunctioning of beta cells of islets of Langerhans causes deficient production of insulin in the pancreases which eventually results in type 1 diabetes. Pancreases transplantation is a complex and risky procedure, also it requires Immunosuppression Therapy.

Producing spheroids of insulin progenitor cells inside biomaterial particles such as mucin best resembles physiology of the pancreatic islets, that can address this problem. Bovine submaxillary (BSM) mucin gels made from culture media can grow cells inside it, these cells will aggregate to form spheroids which can secrete and diffuse insulin through the porous gel structure around it.

In this project we have used principles of droplet microfluidics to produce cell encapsulated microparticles. The project includes design and fabrication of novel microfluidic chip to produce cell laden gel microparticles in HFE oil medium. These particles are characterized in droplet microfluidics aspects by measuring droplet sizes, droplet generation rate and Pressure v/s generation rate. We studied flow at Y-Junction and mixing of two different gelling agents (BSM-N and BSM-T) at the serpentine structure. Also, we discussed a method to retrieve gel particles from the emulsion and cell viability, as well as functionality test has been made.

1.2 Motivation

In recent years, diverse industries and institutions embraced microfluidic technology for the creation of products to improve human quality of life. One such
example include droplet microfluidic technology to make microparticle, microemulsion and microencapsulation. A similar microfluidic tool has been developed at Jönsson biomicrofluidic group at KTH, they found an effective method to encapsulate cells in the droplet which could grow into spheroids following recovery from droplet incubation. Crouzier’s Lab at KTH able to prepare crosslinked mucins to use as a biocompatible biomaterial. These advancements inspired us to recreate pancreatic islets by encapsulating insulin producing cells in mucin microdroplet.

1.3 Research Question

Nowadays type 1 diabetes patients inject themselves doses of insulin to their blood stream or some people undergo pancreatic islets transplantation. Despite controlling insulin levels these methods have potential problems which cause various side effects which requires immunosuppresion therapy[2]. We used droplet microfluidics and a functional biomaterial called mucin to solve the above problem. The aim is to grow islets in gel microparticle which can be used in pancreas to regulate insulin levels. In this project, our challenge is to design and fabricate microfluidic chip which can be optimized to best production of cell laden mucin gel particles. Mucin modulate immune mediated foreign body response and reduces biomaterial induced fibrosis[1]. Hence we selected this material to encapsulate insulin secreting cells.
Chapter 2

Theoretical Background

2.1 Droplet Microfluidics

Droplet microfluidics is a technology which involves generation and manipulation of mono-disperse micrometer sized aqueous droplets inside microfluidic chips.

It provides a compartment in which species or reactions can be isolated, they are mono disperse and therefore suitable for quantitative studies, they offer the possibility to work with extremely small volumes, single cells, or single molecules, and are suitable for high-throughput experiments[3].

Large analysis biological research fields like genomics, proteomics, cell biology and biotechnology need high throughput methods. Droplet microfluidics technology is used in these fields, where low volumes reagents used, analysis times are reduced, sensitivity increased and microfluidic chip fabrication cost is low. Droplet microfluidics is used to generate aqueous droplets in a continuous oil phase and these droplets are stabilized by surfactant. Each droplet act as a test tube vessel for micro reagents where chemical and biological experiments can be performed. Droplet microfluidics allows the isolation of single cells and reagents in monodisperse picoliter liquid capsules and manipulations at a throughput of thousands of droplets per second[4].

2.1.1 Types of Droplet Microfluidics

Droplet microfluidics constitutes two methods continuous-flow emulsion-based droplet microfluidics and electrowetting-based droplet (also called discrete droplet or digital droplet) microfluidics.

Droplet formation of the continuous-flow microfluidics which shown in
Figure 2.1 is the result of an emulsion created using two immiscible fluids, including liquid/liquid and gas/liquid systems. Various techniques, such as channel geometry (T-junction or flow-focusing) and dielectrophoresis, were well applied for good control of droplet generation[5].

![Diagram of continuous-flow emulsion-based droplet microfluidics from T-junction and flow-focusing](image)

The formation of droplets is a spontaneous process and is normally a result of shear force and interfacial tension at the liquid–liquid interface[6].

As for the electrowetting-based droplet, an electric field can change the interfacial tension between the liquid and the surface. Activation of the electrodes leads liquid wetting, and switching off the electrodes reverses. The change in the interfacial tension is capable of producing liquid finger and then breaking off from the reservoir to form a droplet[5].

There has been no simple model for droplet size prediction, a large number of experimental and theoretical studies show that the droplet formation is strongly affected by the channel geometry, flow rate, fluid viscosity, and addition of surfactants[7].

### 2.1.2 Fabrication of Microfluidic Device

Microfluidics has seen the rapid development of new methods of fabrication, and of the components — the microchannels that serve as pipes, and other structures that form valves, mixers and pumps — that are essential elements of microchemical ‘factories’ on a chip[8]. PDMS chip fabrication by using silicon mask (prepared from photolithography) and Acrylic chip fabrication by using CNC micromachining are the general methods used in the fabrication of microfluidic devices. These materials and fabrication methods were adopted because they relied on technology already developed extensively in the microelectronics industry[9].
PDMS-based Microfluidic Devices

Due to its simplicity in fabrication, fairly low background fluorescence, and good bio-compatibility the standard microfluidic device used in scientific research both for continuous flow and droplet based microfluidics is produced by replica molding using poly(dimethylsiloxane) (PDMS) silicon rubber\cite{10}. PDMS device has good transparency to UV light and unique elastoplastic property that make it suitable for conformal contact with glass slides and non-planar substrates\cite{11}. These properties make PDMS use regularly in biological applications.

To fabricate PDMS devices, typically a master is produced by standard contact photolithography and a replica is generated from this master by pouring the already mixed PDMS fluid onto these masters, degassing, and heat curing\cite{10}. After this, it is finished by bonding with glass cover through oxygen plasma treatment.

Acrylic-based Microfluidic Devices

The process of creating a microfluidic chip from acrylic is simple and fast. Poly(methyl methacrylate) (PMMA) chips can be rapidly prototyped through micromilling. The desired chip is designed in AutoCAD and exported to the pre-included software that comes with CNC micromachining. From there, the drill bit can be varied to meet the requirements of the design and to channel parameters. Various factors like feed rate and spindle speed rate can be controlled. The drill bit engraves the microchannel and drills holes on the acrylic plate automatically.

2.2 Fluid Physics at the Nanoliter Scale

In order to work with microfluidics, one must know the physical phenomena that dominate at the microscale. At the microscale, different forces become dominant over those experienced in everyday life\cite{12}. Because of scaling, shrinking existing large devices and expecting them to function well at the microscale is often counterproductive\cite{13}. New designs must be made to take advantage of forces that work on the microscale. The effects that become dominant in microfluidics include laminar flow, diffusion, fluidic resistance, surface area to volume ratio, and surface tension.
Reynolds Number

The Reynolds number (Re) of a fluid flow describes its flow regime—laminar or turbulent. Laminar flow is described in detail below. Turbulent flow is chaotic and unpredictable (i.e., it is impossible to predict the position of a particle in the fluid stream as a function of time). The Reynolds number can be calculated by

$$Re = \frac{\rho ul}{\eta}$$  \hspace{1cm} (2.1)

where $\rho$ is the fluid density, $u$ is the characteristic speed of the fluid, $\eta$ is the fluid viscosity, and $l$ is the characteristic length (depends on the channel’s cross-sectional geometry) of the channel. $Re < 2300$, as calculated by the above formula, generally indicates a laminar flow. As $Re$ approaches 2300, the fluid begins to show signs of turbulence, and as $Re$ becomes greater than 2300 the flow is considered to be turbulent[14].

Laminar Flow

At low $Re$, flow will be in parallel streamlines. This regime, occurs when the viscous forces dominate over inertial forces, is called laminar flow. This is typically the kind of regime that occurs in microfluidics. Laminar flow is a condition in which the velocity of a particle in a fluid stream is not a random function of time. Because of the small size of microchannels, flow is almost always laminar[15]. One consequence of laminar flow is that two or more streams flowing in contact with each other will mix by diffusion.

Diffusion

Diffusion lets particles move from higher concentration moves to lower concentration by Brownian motion untill the average concentration of particles becomes constant through out the volume. Diffusion can be modeled in one dimension by the equation $d^2 = 2Dt$, where $d$ is the distance a particle moves in a time $t$, and $D$ is the diffusion coefficient of the particle[14]. Because distance varies to the square power, diffusion becomes very important on the microscale. By maximizing interfaces between two fluids by long microchannels mixing can be performed sufficiently.
Fluidic Resistance

Fluidic resistance in microchannels is governed by a set of equations whose solutions are well known[15]. The flow rate within a microchannel is given by $Q = \frac{\Delta P}{R}$, where $Q$ is the flow rate, $\Delta P$ is the pressure drop across the channel, and $R$ is the channel resistance. The resistance of a circular geometry can be calculated using the formula

$$R = \frac{8\eta L}{\pi r^4}$$

(2.2)

where $\eta$ is fluid viscosity, $L$ is the channel length, and $r$ is the channel radius. The formula for fluidic resistance varies with cross sectional geometry of the microchannel. For a rectangular microchannel with a low aspect ratio (when $w \approx h$), the resistance can be found by

$$R = \frac{12\eta L}{wh^3} \left[ 1 - \frac{h}{w} \left( \frac{192}{\pi^3} \sum_{n=1,3,5} \frac{1}{n^3} \tanh \left( \frac{n\pi w}{2h} \right) \right) \right]^{-1}$$

(2.3)

where $w$ is the channel width and $h$ is the channel height. The resistance of a rectangular microchannel with a high aspect ratio (when $w \ll h$ or $h \gg w$) can be found by

$$R = \frac{12\eta L}{wh^3}$$

(2.4)

Fluidic resistance is used to calculate pressure drops across microchannels.

Surface Tension

Surface tension has significant importance at the microscale. Cohesion between liquid molecules at the interface of liquid and gas results in surface tension. The surface free energy of a liquid is a measure of how much tension its surface contains. When microchannels are used the liquid travels due to capillary action produced from surface free energy.

The pressure generated by a liquid surface with perpendicular radii of curvature $R1$ and $R2$ can be calculated with the Young-Laplace equation given below

$$\Delta P = \gamma \left( \frac{1}{R1} + \frac{1}{R2} \right)$$

(2.5)

where $\gamma$ is the surface free energy of the liquid[14].
2.3 Mucin- A Functional Biomaterial

Mucins are a family of large glycoprotein polymers expressed both as cell membrane tethered molecules and as a major component of the mucus gel, when secreted by the goblet cells of the epithelium[16]. Mucus gel is a multifunctional material that hydrates, lubricates and forms selective barriers that protect epithelium[1].

![Image](image.png)

*Figure 2.2: Image shows the advantages of using mucin for cell encapsulation.*

Each of those properties has a peculiar role in making mucin a good biomaterial such as a) Barrier Properties: it acts as a barrier to pathogens to form stable coating b) Hydration: forms Hydrogen bonding and Protects from dehydration c) Lubrication by its structure d) Bioactivity: it is bioactive towards microbes and mammalian cells e) Dynamicity: it has good rheological Properties[16].

2.3.1 Bovine Submaxillary Mucin(BSM-N and BSM-T)

BSM molecules are engineered by adding functionality like Tetrazine(Tz) and Norbornene(Nb) forming BSM-Tz and BSM-Nb respectively. They form robust Muc-gels by undergoing crosslinking reaction.
These conjugated BSM gel components are prepared by reacting Tz and Nb amine derivatives with activated carboxylic groups of mucins[1].

2.4 Type 1 Diabetes

Diabetes is prevalence has been rising more rapidly in middle and low income countries. Diabetes is a leading cause of blindness, kidney failure, heart attacks, stroke and lower limb amputation[17].

Diabetes is caused either due to pancreas not producing enough insulin or body cannot use insulin effectively. In type 1 diabetes, the body’s own insulin production has completely ceased. For some unknown reason, the body’s immune system attacks and destroys the insulin-producing cells in the pancreas, which eventually leads to total insulin deficiency[18].

Pancreases consist of Islets of Langerhans, each islet has alpha, beta and delta cells. Beta cells are responsible for the production of Insulin thereby blood sugar level is regulated. But it is still unknown that the immune system which is used to defend the body against infections attacks and destroys its own beta cells[18].

Figure 2.3: Chemical structure of BSM-T and BSM-N and their crosslinking reaction to form gel[1].
Chapter 3

Materials and Methods

3.1 Materials

3.1.1 Software

<table>
<thead>
<tr>
<th>Software</th>
<th>Usage</th>
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<tbody>
<tr>
<td>AutoCAD2019, (3.2.0.63), Autodesk Inc</td>
<td>Design of 2D microfluidic circuit</td>
</tr>
<tr>
<td>Solid Edge ST9, Siemens PLM Software</td>
<td>Design of 3D model and Drawings</td>
</tr>
<tr>
<td>Fiji (ImageJ 1.52i), Wayne Rasband, National Institute of Health, USA.</td>
<td>Image Processing</td>
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<tr>
<td>AmScope 3.7</td>
<td>Imaging software</td>
</tr>
<tr>
<td>Autodesk ArtCAM Premium 2017, Autodesk Inc.</td>
<td>CNC milling</td>
</tr>
</tbody>
</table>

*Table 3.1: List of softwares used in this Project.*
3.1.2 Devices and Machines

CNC Milling Machine: Roland Modela MDX-40A

3D Printer: Ultimaker2

Optical Microscope: Olympus IX53 and Olympus IX51

Fluorescence Microscope: Nikon Eclipse Ti

Digital microscope: AmScope

Programmable Pressure Controller: PC 20

3.1.3 Reagents

<table>
<thead>
<tr>
<th>Product</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFE-7500 Oil</td>
<td>3MTM NovecTM 7500 Engineered Fluid</td>
</tr>
<tr>
<td>Surfactant</td>
<td>1% PEG-PFPE amphiphilic block copolymer surfactant (Ran Biotechnologies)</td>
</tr>
<tr>
<td>BSM-N and BSM-T</td>
<td>Crouzier’s Group, Albanova</td>
</tr>
<tr>
<td>Min6m9 Cell Line</td>
<td>Crouzier’s Group, Albanova</td>
</tr>
<tr>
<td>Alcian Blue</td>
<td>Sigma-Aldrich, Alcian Blue 8GX</td>
</tr>
<tr>
<td>Hepatocytes (HepG2 cell line)</td>
<td>Received from Vamakshi Khati, Scilifelab</td>
</tr>
<tr>
<td>J82 Colon Cancer Cell Line</td>
<td>Received from Sandra Jernström, Karolinksa Institute</td>
</tr>
<tr>
<td>DMEM Media and Fetal Bovine Serum (FBS)</td>
<td>Gibco</td>
</tr>
<tr>
<td>Hoechst 3334</td>
<td>Life Technologies Tm, C47198</td>
</tr>
<tr>
<td>Propidium Iodine</td>
<td>Life Technologies Tm, C27858</td>
</tr>
<tr>
<td>Calcein AM</td>
<td>Sigma Life Science Tm, 56496-50UG</td>
</tr>
</tbody>
</table>

Table 3.2: List of reagents used in this Project
3.2 Methods

3.2.1 Analytical Simulation of Flow Parameters

It is well known that physics of microfluidics follows Newtonian mechanics. In this project before going to fabrication, fluid flow is studied for channels of microscale dimensions. These flows depend on various parameters like channel resistance and Pressure applied across the channel, which results in corresponding flow rate at the outlet.

![Figure 3.1: Dependence of aqueous channel resistance with its dimensions.](image1)

![Figure 3.2: Dependence of HFE oil channel resistance with its dimensions.](image2)

Before fabrication of any chip, we need to design the microfluidic device for specific operational pressure and flow rate we are looking for. This can be done by calculating resistance offered by microchannels. In figures 3.1 and

![Figure 3.3: Resistance offered by different channels in droplet generator.](image3)

3.2 you can see dependence of resistance with other two parameters such as radius and length of channels. We can find outflow rate analytically by using
these graphs when we know pressure drop at the circuit. The Resistance varies with the viscosity of fluid flowing in it apart from channel dimensions.

### 3.2.2 Design and Fabrication of Droplet Generator

Droplet generation device is prepared by Four steps

1) Calculation of dimensions of channels and capacity of reservoirs for the output pressure and flow rate required.

2) Design of microfluidic channels (the lower part of the chip) in AutoCAD software and design of 3D model (upper part of the chip) for reservoirs holders and an outlet port in SolidEdge software.

3) Fabrication microfluidic channels through CNC machining and 3D printing of the upper part of the chip via 3D printer.

4) Assembling all parts to make a finale chip.

![Figure 3.4: Schematic view of droplet generator.](image)

At first various design of microfluidic circuits for droplet generation is made in AutoCAD, then the finale circuit is coming out from improving the design by adding/removing various functional elements, dimensions and position of the elements.
Design improvement is done in different stages as different challenges faced along the way to produce good droplets. In figure 3.5 showing some of the earliest designs, these designs are improved to make them better and working circuit which you see in figure 3.6.

In these circuits consist of different elements like oil channel, aqueous channel (later mucin channel), common channel, inlet and outlet ports. Mass transport in microfluidic devices is generally dominated by viscous dissipation and inertial effects are generally negligible. However, there will be resistance to flow at sharp turnings, hence two oil channels which come out from same
inlet are designed in a curved shape to reduce the pressure loss at the turning and to facilitate smooth flow of the fluid. There are two inlets for mucin channel as there are two different components which are to be equally mixed to make mucin droplets. All these above elements considered while designing including dimensions of the circuit. There requires a design modification for the inlet channel of BSM-N as it is high viscous than BSM-T, where the length of the inlet channel is increased according to the values of viscosity by using mathematical formula below.

\[
\frac{8\eta_1 L_1}{\pi r^4} = \frac{8\eta_2 L_2}{\pi r^4}
\]  

Equation 3.1 is used to calculate the extra length which is required for low viscous component from the inlet chamber.

Designed circuit is transferred to CNC machine where the circuit is printed on PMMA chip automatically, and the various parameters like feed rate, spindle speed, plunge rate and tools specifications are controlled through AutoartCAM software. The printed circuit on PMMA is shown in the figure.

The top of the device is 3D printable part which consists of reservoirs to hold HFE oil and water or mucins, droplet generation view section also the
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Figure 3.8: Top view of microfluidic circuit design for water droplet generation.

Figure 3.9: Isometric view of microfluidic circuit design for water droplet generation.

inlet and outlet ports is designed in SolidEdge software. You can see larger reservoirs for water and smaller reservoir for mucin as it is expensive and used in lesser quantities. The inlet ports for mucin are moved to center which was at the edge in the water reservoir. Figure 3.6 and figure 3.7 shows completed designs for water and mucin cases. The designed file is transferred to the 3D printing machine which prints by using PLA. Once the PMMA circuit chip and PLA top body are ready then the next step is to assemble them.

This is done by using two different tapes, one is double sided adhesive tape (Tesa tape) which is used to combine the top body and chip. Inlet and outlet holes are made on this tape, so that fluid can pass through it. And the other is PSA tape is used for closing fourth wall of the circuit. After finishing fab-
Figure 3.10: Top view of microfluidic circuit design for mucin droplet generation.

Figure 3.11: Isometric view of microfluidic circuit design for mucin droplet generation.

Droplet generation can be done three different setups. First, using a pressure controller to draw fluid from the chip on an optical microscopic platform. Second, manual droplet generation with a pipette and third, Droplet generation by the robotic arm with a pipette on an automated platform. In this project first and the second method is employed to produce droplets, but the chip is designed to work in an automated platform as well.

In the first method, a tube from pressure controller is connected to a pipette...
tip, which is covered with a rubber tube at the entry to fit it with the outlet port. Fluid from the reservoirs is drawn out from the channels into the pipette tip, where the droplets and oil are collected. Once droplets are collected a magnetic valve is used to close the pipette tip bottom, so that the fluid doesn’t come out. After removing the pipette from the chip, the oil and droplets are collected in an Eppendorf tube. In the second method, a pipette is used to draw fluid from the chip where the droplets and oil is collected in the tip.

### 3.2.4 Cell Encapsulation, Cell Culture and Cell Staining in Mucin Gel Microparticles

**Cell encapsulation**

Cell encapsulation in mucin gel microparticles involves three steps as explained below

a) Process of transforming confluent cells to single free cells: 1. 3 of corn-ing flasks (cell culture flasks) containing Min6M9 cells taken and from the incubator and opened them in the hood. 2. Washed with PBS (Phosphate-buffered saline) solution to remove floating cells. 3. The used detaching solution containing accutase/trypsin to remove cells from the surface and made free by weakening interaction(protein interaction between cells and surface) between cells and surface as well as between cells each other. 4. Added FBS (Fetal bovine serum) solution containing 1% antibodies to culturing flasks and incubated. 5. After incubation for approximately 30 mins cells taken from
flasks and checked under a microscope to ensure cells are single free cells or not.

b) Cell counting and calculation for the required concentration of cells in mucins: 1. cell solution taken from a flask and added to a falcon tube, then mixed with a pipette to make a uniform solution. A small amount of cell solution taken (10ul) and spread out on a chamber of a cell counting chip. 2. Cell counting chip is placed on the microscope stage and counted no of cells in 16 squares of the grid on a computer display which is connected to the microscope. 3. Solution has taken in 2 tubes and centrifuged. cells collected from each tube and added to 150ul of BSM-N and BSM-T each respectively.

c) Min6M9 cells encapsulation in mucin droplets, retrieval and incubation: 1. BSM-N and BSM-T containing cells added to inlet reservoirs on the chip and a 2% surfactant HFE isoel is oil is used and droplets are made manually by using Pipette(droplet images were taken after this step). 2. Droplets are placed in oil for 30 mins(gelation time), then retrieved and added to fresh media(images taken after this step) and kept for incubation.

Cell Culture

Cell culture is performed for cell analysis such as cell growth and viability, Cell metabolic activity and also the production of insulin from the cells. To make the cell grow several conditions such as adequate temperature, a supply of oxygen and nutrients and low concentration of toxic metabolites. In our project, we have transferred cell laden droplets to a corning flask and incubated them in DMEM nutrient media(Constitutes FBS) to grow cells. Culture media will be replaced by fresh media every two days once and this is done periodically to grow them completely in microgel particles.

Cell Staining

Cell staining is a technique used for visualizing cells under the fluorescence microscope. By using different stains one can see cell membrane, nucleus and entire cell. In this project cell staining mainly used for testing the viability of cells in droplets. In this project we have used three different stains a) Hoechst 3334 is a fluorescent nuclear stain that is used to stain DNA in cells[19]. b) Propidium Iodine is a red fluorescent stain commonly used to detect dead cells in a population. c) Calcein AM is green fluorescent stain which is used for staining every living cells. 1 uL of each stain is added to 1 mL of media containing cells. Then transferred to a Petri Dish and incubated for 15 minutes before visualizing them under fluorescence microscopy.
Chapter 4

Results

4.1 Droplet Size and Droplet Generation Rate Measurement

Figure 4.1: Images of droplets produced at A)2kPa, B)5kPa, C)10kPa and D)15kPa.
The size distribution of droplets at different negative pressure applied at the outlet port. Images were taken in optical microscope under 4x magnification and then measured droplet dimensions by using Image J software.

After the generation of water droplets in oil, it is observed that droplets are not exactly of the same size. In order to check differences in size for over a number of droplets, a size distribution has been made at by applying negative 2, 5, 10 and 15 kPa pressure at the outlet port. Images of droplets taken in optical microscope under 4x magnification and then measured droplet diameter by using Image J software. In figure 4.1 shows water droplets are produced at four different negative pressure values. It is found that the size of the droplets is not constant, and it is distributed to a range of values in the microscale range. We can observe in figure 4.2 that trend in the distribution is a bell curve indicating droplets have taken range of diameter. As water is a clear and simple system we evaluated the device by using water as a model before generating mucin gel particle generation.

We can see the rise in droplet generation rate from 2kPa to 10kPa in the figure 4.3 and it is expected to increase but the generation rate at 15kPa is de-
increased. During the experiment, there was less fluid in the inlet reservoirs and at high pressure atmospheric air is entered through inlet ports. Droplet generation rate is measured by counting droplets produced in 600 photos taken in 6 seconds at each pressure value.

### 4.2 Flow Profile of Fluids in Microchannels and Mixing

The microfluidic chip is intended to produce droplets of equal concentration from two different inlets at the water or mucin droplets. It is confirmed from the microscopic image at Y-junction that two fluids are coming at equal volume which is the desired flow to conduct further experiments.

It is found that at Y-junction flow is not stable at low pressure values and the flow in Y-junction is stable above the negative pressure of 4kPa. Flow profile at Y-junction can also be tuned by varying fluid column at reservoirs. Found good gelation of mucin gel particle indicating near the equal flow of both the components at Y-junction.
Figure 4.4: Flow profile of two different coloured fluids flowing at sections in the microchannel

Dye Mixing Test

Figure 4.5: Droplets produced by using two dyes at two different inlets
It is essential that droplets must contain equal volumes of both fluid from aqueous inlets with proper mixing.

To investigate this issue a food dye mixing experiment was made, where diluted red and green food dyes at two different inlets are used. It is observed that most of the droplets are brown in colour indicating mix of red and green dyes equally. Also, there are unmixed droplets which are pure red and green in colour. As you see in figure 4.6 the unmixed droplets are in a small fraction. Among 576 droplets 513 (89.06%) droplets are brown, 52 (9.02%) are green and 11 (1.90%) are red in colour. At the beginning of the droplet generation in the Y-junction one of the dye comes before the other and fails to mix with the other dye hence few droplets are not mixed.

**Fluorescent Mixing Test**

In order to see the components of droplets are mixed or not, a Red Fluorescent beads of 2um and a green Fluorescein 10 micromolar is used in two different inlets and droplets are produced. A droplets holder is designed and fabricated which helped to hold the droplets to stay still so that florescent images can be taken. The images represent that Fluorescence material from both the inlets imparted to the total constituent of the droplet. It is found that the components of droplets are distributed all over the droplets as we observe qualitatively.

![Graph showing the number of droplets mixed and not mixed.](image-url)
Figure 4.7: Images ‘a’ and ‘b’ showing red fluorescent beads and fluorescein respectively and image ‘c’ is a bright field image.

4.3 Generation of Mucin Gel Particles

Mucin gel droplets are produced from the hand pipette and observed good gelation within 30 mins after gelation. The mean diameter of mucin gel droplets is 262um and the standard deviation is 11.9.

Figure 4.8: Mucin gel microdroplets.
These values are given by measuring 58 droplets. In figure 4.9 we can’t see the exact trend but 15kPa and 5kPa droplets have low mean diameter than 2kPa. Mucin particles are produced from pipette hence these mucin droplets are formed at pipette pressure. In figure 4.9 we can see that the size of mucin droplet is less than water droplets produced at 2kPa, 5kPa and 10kPa.

Figure 4.9: Size distribution of water droplets produced at different pressures with mucin gel droplets.

Retrieval of Mucin Gel Particles from the Oil Phase

Once after generation mucin droplet would stay inside till gelation to form mucin gel particles. Mucin gel particles are functionalized for culturing cells inside, we must have to transfer it to media or other aqueous phase.

To achieve this we have used oleophilic membrane which only transfers oil through but not the gel bead as shown in figure 4.10. By this we could achieve successful retrieval of gel particles from the oil phase.
4.4 Encapsulation of Cancerous Cell Lines in Mucin Gel Particles

Before encapsulation of insulin secreting cells we have tried with encapsulation of two different cancerous cell lines called J82 cell line which is a colon cancer cells and the other is HepG2 cell line which is liver cells called hepatocytes. we found some interesting inference which was helpful to improve experiments for encapsulation of insulin secreting cells.

4.4.1 Encapsulation of J82 Cell Line in Mucin Gel Microparticles

We were able to encapsulate J82 cells in mucin droplets as we see in figure 4.11. Just after generation, we could see that cells were staying inside droplets, once after it is subjected to one day incubation in an eppendorf tube. During the live/dead staining it is found that cells were traveling out from gel particles. The figure 4.12 was inferring that cells can easily move out due to low gelation strength. It is observed to be crosslinking in the gel is not proper.
4.4.2 Encapsulation of HepG2 Cell Line in Mucin Gel Microparticle

In this encapsulation experiment, we have used optimized mucin hydrogel which shown better cross-linking of two different components to form good gelation than previous version of mucin gel beads. We could see from figure 4.13 are cell laden mucin droplets image taken just after generation and in the figure 4.15 shows live staining of retrieved droplets incubated for a day, here we can notice that cells were present inside hydrogel particles and image indicates that there is considerable viability of cells inside gel. In this case we overcome previous problem that cells moving outside due to bad gelation.
4.5 Encapsulation of Min6M9 Cells in Mucin Hydrogel Droplets

Min6M9 cells are mouse pancreatic cells which can form Islet structure in mucin gel made of DMEM culture medium and secrete insulin in macro hydrogels like Mucin and Alginate. Here in our project we used these cells to encapsulate in mucin hydrogel which has a size around 300 µm. The photos taken after generation, found that cells are aggregated, scattered and resided at all possible positions in different planes.
4.5.1 Experiment 1: Encapsulation of Min6M9 cells in Mucin Gel Microparticles and Growth of Cells

Min6M9 cell laden mucin gel beads containing cell concentration of $3.6 \times 10^7$ cell per ml is incubated for 6 days.

![Image 191x511 to 376x649]

*Figure 4.16: Min6m9 cells in mucin microdropets.*

![Image 99x319 to 468x457]

*Figure 4.17: Min6m9 cells in mucin microparticles after 3 days incubation.*

*Figure 4.18: Min6m9 cells in mucin microparticles after 6 days incubation.*

In figure 4.16 shows that a high density of cells encapsulated inside mucin gel droplets. After retrieval these gel particles subjected to 3 days of incubation and it is observed that in figure 4.17 cells were growing outside the gel particles and moving towards the edge of the droplet. We could see that cells forming clusters at the edge of the particles after 6 days of incubation. These images 4.17 and 4.18 gave an indication of growth of cells for a period.
4.5.2 Experiment 2: Encapsulation of Min6m9 Cells in Mucin Gel Microparticles and Testing their Viability

Min6M9 cell laden mucin gel beads containing cell concentration of $1.38 \times 10^6$ cell/ml with viability 95% are produced.

Figure 4.19: Min6m9 cells in mucin microparticles.

Figure 4.19 shows cell laden gel beads just after generation. These droplets are quite poly dispersed. Also, cells were moved to the edge of the droplet. After retrieval these cell laden gel particles are incubated for 9 days. Live/dead staining is done after 3 days and 9 days.

Figure 4.20: Live staining of cells in mucin microparticles after 3 days.

Figure 4.21: Live staining of cells in mucin microparticles after 9 days.

Figures 4.20 and 4.21 indicates that cells are live but after 9 days of incubation cells did not survive. One reason for this is that DMEM media which
is used to cultivate these cells did not contain FBS (Fetal Bovine Serum).

**4.5.3 Experiment 3: Encapsulation of Min6m9 Cell inside Mucin Gel Particles and Growth of Islets like Structure Inside Mucin**

This experiment was conducted in Albanova by moving our microfluidic device to there. Min6M9 cells encapsulated as we see in figure 4.22 and placed in PBS after retrieval to take images and which is shown in figure 4.23.

![Figure 4.22: Min6m9 cells in mucin microdroplets.](image1)

![Figure 4.23: Min6m9 cells in mucin microparticles in PBS after retrieved from oil.](image2)

These cell laden particles are incubated for 30 days and took images of cells after 11, 15, 21, 28 and 30 days. It is observed that after 15 days Islet-like tissue started to appear in the hydrogel particle which represents growth of cells in mucin hydrogel as shown in figure 4.24.

Subsequently, we could able to observe islet-like structures forming till day 30. We have noticed that cells which are free in the middle of the gel particles are tend to form islets than the cells which are aggregated at the edge of the droplet. When cells aggregate they start lacking nutrition also they reach high confluence, After that they stop growing and leads to necrosis.
Figure 4.24: Growth of Min6m9 cells in mucin microparticles from day 11 to day 35.

4.5.4 Experiment 4: Encapsulation of Min6M9 cells and Growth of Islet like Structure

Min6M9 cells were encapsulated in mucin hydro gel droplets at science for life laboratory and carried away to Albanova laboratory for cultivation of cells. Cells inside mucin hydro gel cultivated for 35 days and found to form islets inside them. In this as shown in figure 4.25 cluster of cells are reduced and we could able to see more islet from the previous case. While encapsulation in this experiment we have used pressure controlling device to draw oil and mucin droplets from microfluidic device instead of pipette. In this case encapsulated cell density was low compared to previous encapsulation and cells are quite free and distributed inside gel particles.
4.6 Automated Production of Droplets

Droplet microfluidic device can be used in fluid handling robot for automated droplet production. With the help of group member, we have written python code for fluid handling robot for automated production of droplets. Automated production helps in mass production of droplets automatically without human aid and the other use is through automation we can able to eliminate suspension of cells at the edges of the droplets. If the suspension of cells in mucin microparticles then the number of islets formation may increase.
Chapter 5

Discussion

In the fabrication method we have choose PMMA (Poly methyl methacrylite) chip as it is inexpensive and easy to prepare and optimize design over other methods.

Size distribution of droplets produced at different values of negative pressure was recorded, there was no exact trend in the size of droplets in accordance with pressure. Size of the droplets formed at 15 kPa and 5 kPa is lesser than size of the droplets produced at 2 kPa.

It is found that there is no trend in size of droplets with pressure but at higher negative pressure like 15kPa and 5kPa has produced droplets of lower sizes than 2kPa.

Mixing is one of the primary element considered in the design of microfluidic device but as we observed from the data mixing was good in water droplets. Whereas, Mixing serpentine was not implemented in mucin droplet generator as mucin is viscous and any remaining mucin after generation may form gel and block the chip. Also mucin was able to mix and form gel even in the absence of serpentine channel.

Although our project was aimed to encapsulate insulin producing cells we encapsulated cancerous cells in order to study the behaviour of cells and to check growth of the cells in mucin hydrogel.

We were able to encapsulate Min6M9 cells inside mucin hydrogel and observed islet like structures forming inside the gel. Cells that are free at the center can form islets but the cells that are clustered at the edge couldn’t form islets. One of the reason that clustered cells can’t form islets is that they lack nutrients from culture media than the cells which are free. The other reason is cells stop growing when it reaches high confluence.
Chapter 6

Conclusions

Droplet microfluidic device which is fabricated in this project can generate cell laden mucin gel microparticles. Min6M9 cells were able to form Islets-like structures inside gel micro particles, which indicate that mucin hydrogel supports the growth of mouse pancreatic cells inside it. The project can be carried further to test biological activity and production of insulin from the cells.
Bibliography


