Cell Membrane Perturbation and Micro-confinement Induced Variation in Giant Plasma Membrane Vesicles

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by

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I the principal supervisor below signed, after checking the dissertation mentioned above and the official record books of the student, hereby state my approval of the dissertation submitted in partial fulfillment of the requirements of the degree of Master of Technology in Biotechnology at National Institute of Technology Rourkela. I am satisfied with the volume, quality, correctness, and originality of the work.

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Principal Supervisor

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May 18, 2016

Supervisors' Certificate

This is to certify that the work presented in this dissertation entitled *"Cell Membrane Perturbation and Micro-confinement Induced Variation in Giant Plasma Membrane Vesicles"* by "*Yamini Yogalakshmi*", Roll Number 214BM2033, is a record of original research carried out by her under my supervision and guidance in partial fulfillment of the requirements of the degree of *Master of Technology* in *Biotechnology and Medical Engineering*. Neither this dissertation nor any part of it has been submitted for any degree or diploma to any institute or university in India or abroad.

Indranil Banerjee
Principal Supervisor

Dedicated to my loving Mom, Dad and brother

Declaration of Originality

I, Yamini Yogalakshmi, Roll Number 214BM2033 hereby declare that this dissertation entitled *"Cell Membrane Perturbation and Micro-confinement Induced Variation in Giant Plasma Membrane Vesicles*" represents my original work carried out as a postgraduate student of NIT Rourkela and, to the best of my knowledge, it contains no material previously published or written by another person, nor any material presented for the award of any other degree or diploma of NIT Rourkela or any other institution. Any contribution made to this research by others, with whom I have worked at NIT Rourkela or elsewhere, is explicitly acknowledged in the dissertation. Works of other authors cited in this dissertation have been duly acknowledged under the section *"References"*. I have also submitted my original research records to the scrutiny committee for evaluation of my dissertation.

I am fully aware that in case of any non-compliance detected in future, the Senate of NIT Rourkela may withdraw the degree awarded to me on the basis of the present dissertation.

May 18, 2016
NIT Rourkela

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Abstract

Giant Plasma Membrane Vesicles (GPMVs) are cell-membrane model systems; characteristically they are fluid filled irreversible membrane blebs. Thus they are used to decipher the membrane dynamics. In this study we generated GPMVs with the help of chemical vesiculants that induce irreversible blebs from four different cell lines namely; HaCaT (immortalized human keratinocytes), Hela (cervical adenocarcinoma), HT-29 (colon cancer) and MG-63 (osteosarcoma). The experiments were carried out both under macro conditions and micro conditions. Different cell lines showed distinct patterns in vesicle generation in both macro and micro conditions.

MG-63 cells were taken up for further studies to identify the role of cytoskeletal reorganization in vesicle generation. In order to analyze this; Cytochalasin-D, an actin polymerization blocker and Methyl-β-cyclodextrin (MβCD), a cholesterol depleting agent were used to disrupt the lipid raft arrangement. Using fluorescence microscopy, the growth kinetics of the vesicles was studied with the help of lipophilic dyes such as DiI and DiO. It was observed that highest number of vesicles and vesicles with largest diameter were obtained when MG-63 cells were treated with MβCD. DPH-Anisotropy was calculated to identify the membrane fluidity and it was found that MβCD treated cells were exhibiting higher membrane rigidity due to the absence of cholesterol.

In order to assess the phase partitioning of the plasma membrane, confocal microscopy was done to observe the lipid ordered state (L_o) and the lipid disordered state (L_d). Lipid profiling was done after lipid extraction and extracts were used further for FTIR spectroscopy, phosphorous assay, and cholesterol estimation. Upon optimizing the culture conditions for GPMV synthesis the cells were grown in microfluidic cell culture platforms.

The higher the channel height the greater the number of GPMV per cell were obatained, however in the channel of 100μm height, number of GPMV per cell was reduced pertaining to GPMV coalescence. Based on optimization data, it was inferred that synthesis of GPMV and its qualitative features can be controlled using the channel height of the microfluidic platform. The present study may find its application in designing a new membrane model and the know obtained from this study can be explored for generating new therapeutic packages.

Keywords*: *Giant Plasma Membrane Vesicles; MβCD; Cytochalasin-D; micro-confinement; anisotropy; lipid profiling

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Chapter 1

Introduction

Giant Plasma Membrane Vesicles

Giant Plasma Membrane Vesicles (GPMVs) also referred to as blebs, are fluid-filled membrane-bound projections originating from the cell membranes of living cells both eukaryotic and prokaryotic in origin. They develop when the cortical actin present at the internal periphery of the cell membrane can no longer retain the intracellular pressure; this in turn results in the dissemination of the plasma membrane from the cytoskeleton. During cell division, locomotion, death, signal transduction and apoptosis these blebbing pattern can be observed, which in certain cases get cut off to generate vesicles (1). GPMV growth and formation are both broadly determined by the cell through regulation of the plasma membrane dynamics and cortical actin reorganization by key cellular mechanisms. A key characteristic of these natural GPMVs are their reversible property since they are initiated by a protein-mediated, momentary, asymmetric loss of cortical actin organization and immediately reorganize back into the cell. Whereas, irreversible GPMVs are generated from processes such as burn, hypoxia, infection or inflammation where damage triggered stress serves as the driving force for cortical actin and plasma membrane separation. These irreversible GPMVs are a spontaneous cellular response which is necrosis mediated and are also referred to as cell blisters.

Reversible blebbing has been studied extensively, but not much is known of the underlying mechanism of irreversible GPMVs. Irreversible GPMVs are the key aspects of this study. Irreversible GPMVs are excellent membrane model entites and can also serve as

bodies used to better understand identified as potential biological membrane models that harbor

Giant Plasma Membrane Vesicles have therefore been identified as potential biological membrane models that harbor membrane associated proteins in native form and simultaneously show phase separation supporting the existence of lipid raft hypothesis. Studies have also been carried out to understand the functional importance of the lipid raft in cell plasma membrane in cellular processes and signaling mechanisms as a result of environmental changes and modifications (16). However, their limitations that arise due to the usage of chemical vesiculants during isolation demand the need for improvised methods of vesicle isolation to better derive ideal membrane models representative of functional lateral heterogeneity.

Vesiculating Agents

Studies carried out by scientists previously have mostly dealt with complete destruction of the cells either by physical or chemical vesiculants. Physical methods such as laser irradiation, osmotic swelling have also been utilized however chemically induced vesicles have been studied broadly and optimized as better biological plasma membrane models. As a result the most prominent limitation of these irreversible GPMVs is the chemical vesiculant induced covalent modifications and interactions.

The broadly used combination of vesiculants is that of paraformaldehyde and dithiothreitol, which are nonspecific cross-linkers and reducers, respectively. This procedure has circumvented the unintended side effects encountered while using N-ethyl maleimide (NEM), which irreversibly modifies terminal sulfhydryls (typically free cysteine side chains those not engaged in disulphide bridge formation), covalently blocking these groups without crosslinking [3]. Other than the most obvious chemical modifications resulting in vesiculation, numerous cellular processes that occur simultaneously during vesicle isolation and generation protocol due to their complex and nearly impossible mode of prediction, these and to the penultimate demerit of GPMVs.

For instance, the loss of membrane leaflet asymmetry, is distinctly determined by the presence of the anionic lipid phosphatidylserine on the membrane surface. In spite of GPMV membranes being asymmetric to certain extent; membrane associated proteins generally retain their native structure. Therefore it is quite intriguing how phosphatidylserine is greatly exposed on the exoplasmic leaflet, when in comparison to live whole cell plasma membranes.

Phase Partitioning

The lipid raft hypothesis suggest nanoscale arrangement of animal cell plasma membranes pertaining to preferred interactions between membrane lipids and membrane associated proteins. This eventually gives rise to functional lateral membrane domain regions in the plasma membrane. This preferred organization is typically as a result of cholesterol-dependent formation of a liquid-ordered membrane phase (Lo) that can simultaneously coexist with a liquid-disordered phase (Ld) which is predominantly comprised of unsaturated fatty acids and depleted of saturated lipids such as cholesterol/glycosphingolipids. In spite of these interactions, long-range organization has been broadly studied in purified lipid model membranes (i.e. synthetic liposomes which include unilamellar vesicles like Giant Unilamellar Vesicles, Large Unillamellar vesicles and Small Unilamellar Vesicles), the complexity of whole live cells, lack of cuttingedge experimental methods, and the ultimate inability to accurately visualize lipid rafts on cell membranes led to significant debates on both the functional and existential properties of lipid rafts.

Puzzling as it may seem, lipid rafts have been proven with the aid of new emerging membrane models in addition to current technological advances such as super-resolution and electron microscopy, nanoscale spectroscopy, and lipidomics have all resulted in the successful identification of functional domains in lateral biological membranes (10). These domains are subject to lipid mediated phase separation (also referred to as membrane rafts) or by cholesterol-independent protein–lipid and protein–protein interaction, hypothesized to have significant overlap and cooperation between both processes.

The outcome of domain formation is mainly observed to be functional segregation of membrane lipids (11). In whole cells, lipid-driven membrane rafts are difficult to be observed due to the complexity of cells with several organellar interactions and dynamic nature of the lipid membrane (12). Thus, advanced techniques are required for the measurement of domain compositions and properties. Therefore GPMVs are emerging as an efficient alternative for isolation of intact plasma membranes (PMs) retaining the integrity and complexity of native membrane components.

The coexistence of two liquid phases with distinct physical properties and compositions in these naturally occurring membranes provides pivotal evidence for the lipid raft hypothesis (13). Most significantly, GPMVs comprise an intermediate biological membrane model system, combining the compositional complexity and protein content of live cell membranes with the macroscopic phase separation and experimental malleability of synthetic vesicles (14). The most important merit of this model system is that it allows

quantitative measurement of protein partitioning and (potentially) functions in large, stable, well-resolved domains. This possibility has enabled investigators of the structural determinants of raft partitioning and is likely to yield more insights about raft-dependent protein function in the coming years

The lipid raft hypothesis has been debatable over the years but with the advancement of microscopic tools and more significant membrane models has enabled the acceptance of the role and existence of the lipid rafts. The key focus of this review are Giant Plasma Membrane Vesicles one such membrane model used to better understand the complex and dynamic phase partitioning in biological membranes. GPMVs are preferred over GUVs since they retain the complex nature of biological membranes yet simultaneously lack interferences of organellar interactions and other cellular processes.

The discovery of GPMVs generation from various mammalian cell cultures has led to the broad phase coexistence based studies that influence cellular functions and sub-domain distributions, for instance protein partitioning in membranes. Here we go on to discuss how GPMVs tend to be more ideal membrane models when coupled along with robust experimental methods and technology to decipher the underlying mechanism of membrane complexity and functioning.

Microfluidic cell culture platform

Microfluidics is an established multidisciplinary field of research of especially in physics, chemistry, biochemistry, nanotechnology and is now rapidly advancing in the field of biological sciences. This currently advancing field of research is termed as Biomicrofluidics. The tools globally used in Microfluidics are those used in several fields of engineering such as electronic and electrical engineering. Techniques such as photolithography are used to prepare master moulds that are in turn used to generate microfluidic platforms by other methodologies such as soft lithography [1].

Even rapid prototyping is used as an advanced tool for development of precise microfluidic platforms. To be considered a microfluidic device any one dimension of the device has to be in a microscale distinctly. The main features for micro conditions indicates; small in size, small volume, lower energy requirement and effects of micro confinement. The fluidics aspect is brought about due to the microflow of a fluid in the microchannel

Chapter 2

Literature Review

The Bleb

Bleb formation has been observed by intrinsically studying important factors regulating this process; these include microtubule disassembly, increased pressure, and local actin de-polymerization. A minimum of two different types of blebs (types 1 and 2) are developed by various methodologies and a hypothesized third type (type 3) could be observed at the front of live polarized cells with the help of tagging with green fluorescent protein-actin and/or in stained and fixed cells.

Type 1 blebs commonly known as membrane/cortex dissociation blebs are generated by the spontaneous disengagement of the plasma membrane from cortical actin. This causes development of cytoplasmic actin layers that are observed with restriction rings. They can be chemically induced by agents such as the microtubule-disassembling agent; colchicine. Whereas, Type 2 blebs which are commonly known as cortical actin disassembly blebs are generated after disassembly of the cortical actin layer under the influence of latrunculin A. This can be distinguished by the presence of restriction rings without a cytoplasmic actin layer present in a transition zone between the rigid cortical actin layer of the cell body and the disintegrated actin filaments of the bleb.

The third type of bleb (type 3) is confirmed, upon demonstrating an intact cortical actin layer with absence of cytoplasmic actin layer. In addition, no significant relationship between the actin cytoskeleton and the restriction ring has been observed when visualized with the aid of passive cell deformation in micropipettes, which causes an increases

pressure.

For instance, re-polymerization of the cortical actin layer does not always result in bleb readsorption. Once formed, restriction rings do not constrict, indicating that they result from the phenomena considered as isometric contraction. A generalized classification scheme has been generated to match the type of bleb to distinct signals or cell functions. Its application shows that spontaneously blebbing cells form almost exclusively type 1 blebs.

The lipid raft hypothesis

Previously, the lipid raft hypothesis refers to the nanoscale organization of mammalian cell membranes which are fuelled by preferential interactions between specific lipids and proteins that give rise to functional lateral membrane domains. The physicochemical parameters regulated for this organization are mainly driven by the cholesterol-dependent formation of a liquid ordered membrane phase which is observed to coexist with a liquid-disordered phase that is in turn rich unsaturated lipids and depleted of saturated lipids such as cholesterol, glycosphingolipids [2].

In spite of these interactions, their eventual long-range organization have been extensively studied in synthetic lipid model membranes such as Giant Unilamellar Vesicles [2]. The inherent complexity of living cells, lack of competent experimental techniques, and the resultant inability to directly image lipid rafts was the key cause of the significant controversy regarding their functional role [4] and even their mere existence [5].

Whereas, the developments in techniques and methodologies in plasma membrane isolation [6] these include techniques such as nanoscale spectroscopy [10, 11], super-resolution microscopy [12] and electron microscopy methodologies [13], and lipidomics study [14, 15] have all led to the overall definitive observations of the existence of lateral functional domains in biological membranes.

These structures can be initiated primarily either by the basic lipid-driven phase separation which is commonly known as membrane rafts or by the cholesterol-independent protein–lipid and protein–protein interactions, similarly with significant overlap and mutualism between these mechanisms [16]. In general, the eventual existence of domain formation is ideally the functional segregation of membrane associated components. In living cells, lipid-associated membrane rafts are occasionally observable by light microscopy because of the aspect of length and time dependent domain separation are estimated to be in the range of tens of nanometers in size and are dynamically evolving on millisecond timescales.

Therefore, cutting-edge techniques are demanded for the measurement of domain

compositions and their intrinsic properties. Giant plasma membrane vesicles (GPMVs) are therefore used as one such entity for efficient isolation of intact plasma membranes (PMs) which simultaneously maintain the entire diversity of native membrane associated components [17]. The coexistence of two dynamic liquid phases with distinct physical properties [18] and compositions [19, 20, 21] in these natural mimicking membranes provides persuasive evidence for the core theory of the lipid raft hypothesis.

Even more significantly, GPMVs are regarded as an intermediate biological membrane model system, which combine the compositional diversity and protein content of living cell membranes with the evident macroscopic phase separation and experimental malleability characteristic of synthetic vesicles. The key advantage of this model system is that it enables quantitative measurement of protein partitioning [20] and the possibility to function in large, well-resolved, stable domains. This property has allowed the investigation of the structural determinant factors of raft partitioning [22] and is likely to decipher more insights about raft-influenced protein functioning in the years to come.

Bio-membrane models

The evolution of life was without doubt, closely associated to the development of the membrane and its lipid components. After the cell membrane has evolved, the following questions arose; whether the cell constituents could be restricted to a confined space; the ability of nutrients to diffuse in and out, and similarly selective waste removal, at regulated rates; could membranes be established as protective barriers against chemical and biological anitgens; could the role of membrane contacts lead to the maintenance of the integrity and eventually restrict the growth of significant multi-cellular populations; could membrane fusion regulate biological processes such fertilization of gametes and viral attack associated loss of membrane integrity; and could membrane fission help drive the proceeding of cell division during mitosis and meiosis. It is in admiration of the cell membrane that we write this Account.

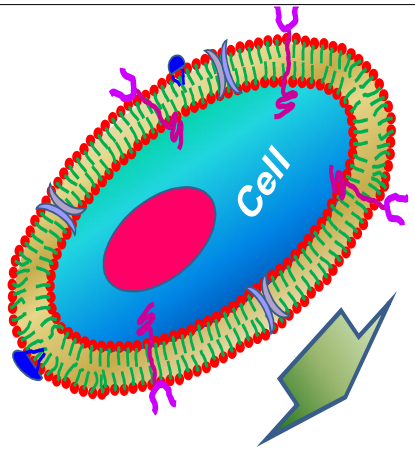

Figure 1 ; Schematic representation of Giant Plasma Membrane Vesicle

Evidently, the primary membrane component which comprises of the lipid population seems mundane and not as interesting compared to other macromolecules such as proteins and nucleic acids. However, this perspective is discriminative because the ordinary lipid generally does not function as a single entity as is the case with enzymes that are proteins in nature or RNA molecules; lipids as a substitute form “molecular communities” through self-assembly. These communities are very dynamic an complex far from their misconceived simplicity. They actively undergo processes such as fission, fussion, endocytosis, and budding; which simultaneously incorporate membrane channels that continuously open and close (altering their conformation); they enable the establishment of pH gradients that drive ATP formation; they comprise of the framework for trapping light energy; they shuttle proteins from organelle to organelle; they are majorly involved in regulating the activity of embedded enzymes; and the constitutive lipid molecules undergo diverse relocations such as flip-flop, rotation, lateral diffusion, and phase transitions. Therefore, the lipid is a particularly lively biological entity which, when aggregated into bilayers, is capable of magnificent chemical and physical processes that are essential to life.

Table 1: Review of Literature

S.No	Title	Key Highlights	Publication
1	Advantages and challenges of microfluiidic cell culture in polydimethylsiloxane devices.	Flexibility of device design, experimental flexibility and control, a low number of cells is required, single cell handling, real-time. On-chip analysis, automation, direct coupling to downstream analysis, reduced reagent consumption, controlled co-culture.	Biosensors and Bioelectronics 63 (2015) 218–23
2	Elucidating membrane structure and protein behaviour using giant plasma membrane vesicles.	**2mM NEM concentration** was optimized in this protocol coupled with PFA/DTT for macrofluidic systems. Since even though used alone PFA/DTT high yield of vesicle is obtained but several artifacts are obtained	NATURE PROTOCOLS Vol.7 NO.6, 1042-1052 (2012)
3	Pulsed-laser creation and characterization of giant plasma membrane vesicles from cells.	This was an initiative for use of physical stimuli for controlled and targeted vescile generation. The vescile contained fragments of F-actin and cytoplacmic content identified by fluorophores namely **C-laurdan** and polystyrene beads.	J Biol Phys (2009) 35:279–295
4	Temperature dependent phase behaviour and protein partitioning in giant plasma membrane vesicles	Phase segregation occurs reversibly below 37^0C, binding of peripheral proteins, such as cholera toxin subunit B (CTB), as well as annexin V, is observed to modulate phase transition temperatures, indicating that peripheral protein binding may be a regulator for lateral heterogeneity in vivo	Biochim Biophys Acta. 2010 July ; 1798(7): 1427–1435
5	Differences in cortical actin structure and dynamics document that different types of blebs are formed by distinct mechanisms	Major factors controlling this process, such as microtubule disassembly, local actin depolymerization, and increased pressure. It was identified that GPMVs are Type 1 blebs (membrane/cortex dissociation blebs)	Experimental Cell Research 277, 161–172 (2002)
6	Non-equilibration of hydrostatic pressure in blebbing cells	protrusive motility in animal cells focus on cytoskeleton-based mechanisms, where localized protrusion is driven by local regulation of actin biochemistry	NATURE Vol 435 19 May 2005
7	Estrogen augments shear stress-induced signaling and gene expression in osteoblast-like cells via estrogen receptor-mediated expression of beta1-integrin.	It was observed that osteoblast-like MG-63 cells were able to withstand shear of **12dyne/cm^2** and were able to mechanosense (such as cytoskeletal reorganization, modulation in gene expression etc)	J Bone Miner Res. 2010 Mar;25(3):627-39

Chapter 3

Objective and Work Plan

Objectives

- Synthesis of Giant Plasma Membrane Vesicles (GPMVs)
- Effect of cell membrane perturbations on GPMV
- Effect of micro-confinement on GPMV

Work Plan

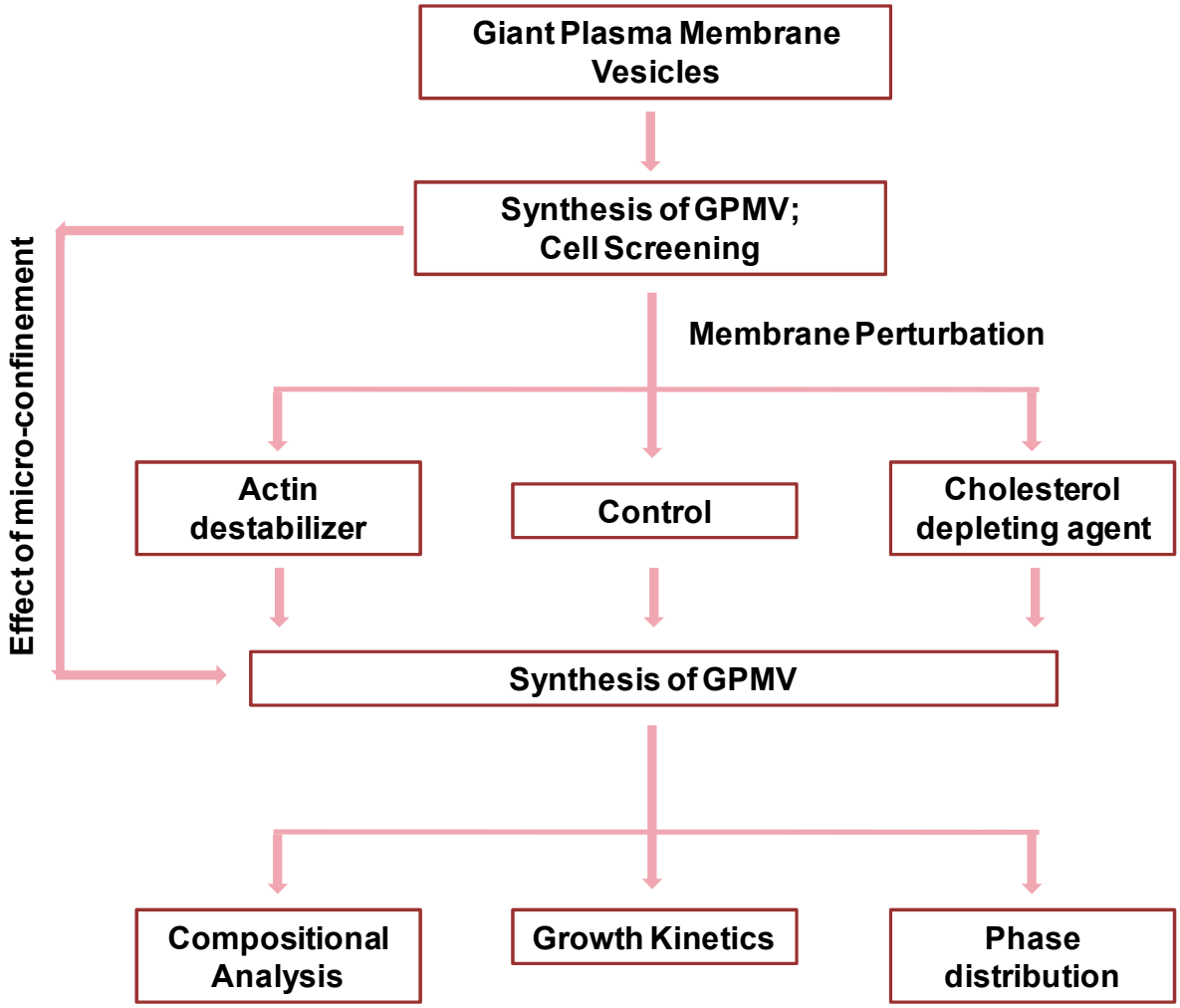

Figure 2; Schematic overview of Workplan

Chapter 4

Materials and Methods

Materials

The cell lines used were obtained from NCCS Pune. The DMEM medium, fetal bovine serum (FBS), antibiotic-antimycotic solution and trypsin-EDTA solution were purchased from Gibco. N-Ethylmalemide, Paraformaldehyde, Dithiotheritol were all purchased from Sigma-Aldrich. 100% ethanol was purchased from MERCK. Polydimethylsiloxane (PDMS) and its compatible curing agent was purchased from Dow Corning. Other chemicals utilized were obtained from Himedia.

Methodology

Microchannel fabrication

The microfluidic channels were prepared using soft-lithography technique. Initially the mold was prepared on glass surface by photolothography using commercially available negative photo resist. Prior to the exposure and development , photo resists were coated on the surface using spin coater. To achieve microchannel of different height, spin rate was varied from 1000rpm to 2500 rpm. Once the molds were prepared, PDMS solution (base : crosslinker 10:1) was poured into it. It was then degassed and soft baked for 4 hrs at 65 ºC. Those PDMS blocks were further fixed on a oxidized glass coverslip. Prior to fixing , inlets and outlets were made in the PDMS channel by mounting 20 gauge blunt end needle

(Figure 2). For further flow experiments , the channels were connected to a syringe pump.

Purification and concentration of GPMV

Synthesis of GPMV from cells were done under the influence of chemical vesiculating agent like DDT /PFA. In brief, cells were cultured in 5%CO_2 incubator at 37º C in DMEM supplemented with 10% FBS. Prior to the experiment, cells were harvested by trypsinization and seeded in six well plate (for macro culture condition) and inside microchannel (micro culture condition). The cell number was adjusted from $5x10^3$ to $1x10^5$ as per requirement. Cells were first allowed to adhered on the surface then washed twice with GPMV buffer (10 mM HEPES, 150 mM NaCl, 2 mM CaCl2, pH 7.4). After washing , the cells were treated with vesiculating (25 mM paraformaldehyde and 2 mM DTT in GPMV buffer) and incubated for 40 min at 37°C. In case of macro culture condition, cell supernatant containing the GPMVs was decanted and stored on ice until further use. In case of micro culture system, the cells were subjected to microflow for detachment of the GPMV and the flow residue were collected. GPMV were further purified by centrifugation at 100*g* for 10 min followed by 20000*g* for 30 min. Finally the GPMV were stored at 4 ºC.

Blocker treatment

The MG-63 cells seeded were treated with various blockers namely Cytochalasin-D an actin polymerization blocker, Methyl-β-Cyclodextrin (MβCD) a cholesterol depleting agent was added. Cells were washed twice with PBS before blocker treatment to eliminate any remnant serum components' inhibitory effect on the functioning of the blockers, 500µL of solution is added to each well of a 6 well plate. These concentrations were optimized based on previous studies carried out on adherent cell lines. Working concentration of 10µM was used for Cytochalasin-D and the time of exposure was fixed as 30 mins and 500 µM was used for MβCD and the time of exposure was fixed as 30 mins. GPMV isolation was carried out after treating the cells with blockers following the earlier mentioned protocol. The blocker solutions were prepared using incomplete DMEM media and stored in -20°C.

Micro-Bradford Assay

This assay was performed to assess the total protein present in the GPMV solution. Standard curve was made using BSA 1mg.mL^{-1} and serially adding 10- 50 µg.mL^{-1} and the total volume was made up to 200 µL. 30 µL of sample was added and total volume made up to 200 µL. Samples were incubated in dark for 10 mins in room temperature and the OD of sample was taken at 595nm using an ELISA plate reader.

Lipid Extraction

To analyze the lipid contents of the GPMV, lipids were extracted from GPMV following the procedure reported by Bligh and Dyer et al. In brief, GPMV containing solution were first mixed with cchloroform and methanol to reach a ratio of 1 : 2 : 0.8. Later 1 volume of chloroform and 1 volume of distilled water were added into the solution. The solution were kept for phase separation for 1 hr. After that the organic phase was collected and the whole process was repeated twice. All the extracted organic phases were pooled together and subjected for evaporation at 40ºC in nitrogen atmosphere.

Phosphorous Assay

To enumerate the quantity of lipid extracted, the total amount of phosphorous was determined from the GPMV lipid extracts following a modified procedure of Fiske & Subbarow from Avanti Polar Lipids (Fiske and Subbarow, 1925). 0- 0.0456 µmoles KH_2PO_4 was used to make phosphorous standards along with the extracted GPMV lipids. Solvent was removed with a stream of N_2 and 90 µL of 8.9 N H_2SO_4 was added to each tube. All samples were heated to 210 ºC in an aluminum heating block for 25 minutes. After which tubes were removed and left to cool for 5 minutes before adding 30 µL of H_2O_2. Tubes were reheated at 210 ºC for 30 minutes, then allowed to cool to ambient temperature before adding 780 µL of H_2O. 100 µL of 2.5% ammonium molybdate tetrahydrate solution was added and again vortexed for 2 mins vigorously. Next 100 µL of 10% ascorbic acid was added and vortexed for 2 mins vigorously. A marble was placed over each tube to minimize evaporation when tubes were heated to 100 ºC for 7 mins. Absorbance of standards was measured at 820 nm using a UV/Visible spectrophotometer and used to generate a linear calibration curve to determine the concentration of phosphorous in each GPMV sample. Predicted concentrations of GPMVs were found.

Cholesterol Estimation

Amount of cholesterol in GPMV was quantified following the protocol reported by by Bowman and Wolf. [22]. For this purpose , total lipid was first extracted from GPMV as per the abovementioned procedure. Prior to the experiments The GPMV lipid extracts and cholesterol standards were dissolved in chloroform. The samples were dried at room temperature. After that 500 mL of ethanol and 500 mL of 'ferric chloride reagent' (0.8 mL of a 2.5% (w/v) $FeCl_3.6H_2O$ solution in 85% H_3PO_4 with 9.2 mL of conc. H_2SO_4) were added

to the dried lipid samples, mixed well and incubated for 30 min. The absorbance was measured at 550 nm.

Labeling cell membranes before GPMV isolation

The seeded MG-63 cells upon reaching 70% confluence in a 6 well plate were washed twice with 1mL PBS and 10µL of dye solution to 1mL of PBS was added to obtain a final concentration of 5µg.mL^{-1}.and was added to cells. The cells were incubated for 4°C for 15min for MG-63 cells. Concentrations and incorporation conditions were optimized for all dyes, although the above conditions were optimized for MG-63 and for the dialkylcarbocyanine (i.e., DiO, DiI and so on) dyes used here. Followed by this 1mL of GPMV buffer was used to wash the cells twice. After which 1mL of buffer containing vesiculation agents in GPMV buffer were added to the washed MG-63 cells. To induce vesiculation the vesiculant treated cells were incubated in 37°C and 5% CO_2. The cell culture system was not kept on a shaking which would generate increased cell debris when compared to the static system.

Fluorescence imaging of GPMVs for growth kinetics

The synthesis pattern of the GPMV from cells was visualized after MG-63 cells were labeled with fluorescent lipophilic dyes. For this study DiO and DiI was used and prior to labeling. Upon immediate addition of GPMV buffer with vesiculating agents MG-63 cells were viewed under the fluorescence microscope and time dependent photographs were taken. Similar methodology was used to observe the GPMV growth pattern in blocker treated and fluorescently labeled cell. Number of GPMV per system was counted for each sample and the temporal manner of GPMV synthesis pattern was understood over the timespan of 40 mins.

Confocal Microscopy

The raft phase was studied using this by placing the GPMVs in BSA coated wells and microchannels to visualize using GPMV. Pre-labeled GPMV was used for this study.

Chapter 5

Results and Discussion

Cell Screening

Four different cell lines were used to assess the cell dependent nature of GPMV synthesis and therefore to identify the most suitable adherent cell line for efficient GPMV synthesis. MG-63 cells were selected for further studies due to their well spread nature and ability to generate high yield of GPMVs and fewer cellular debris were encountered.

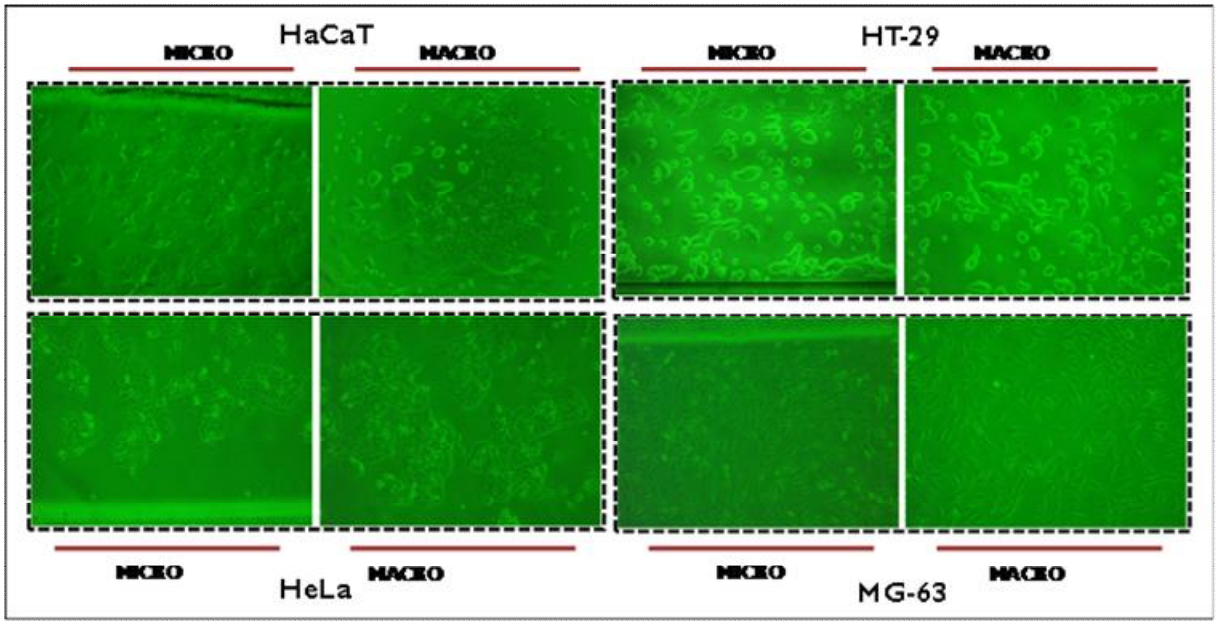

Figure 3; HaCaT, HT-29, HeLa and MG-63 cells grown in both microfluidic and macro conditions

They occupied higher cellular surface area and GPMV synthesis is also governed by adherent cell nature. In order to better identify any significant differences in cell adhesion between both systems similar culture parameters were maintained and the cellular area was calculated with respect to the available are for cell adhesion.

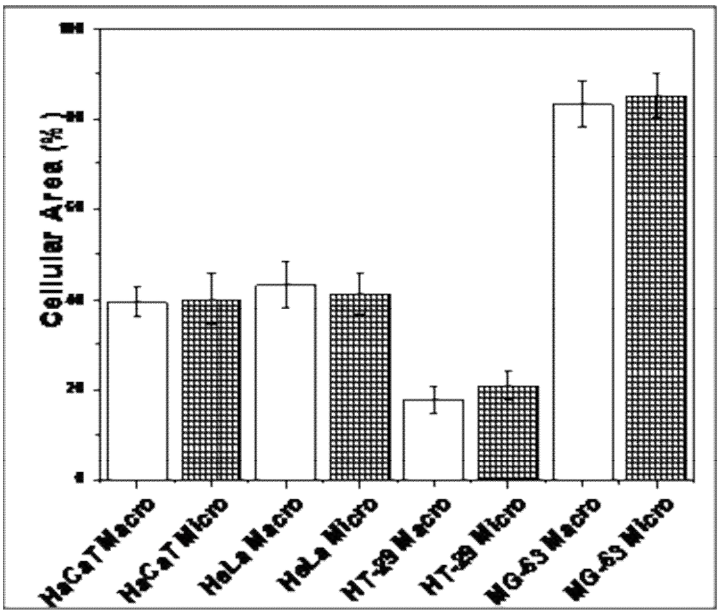

Figure 4 ; Graphical representation of cellular area comparison between macro and micro cell culture systems using four cell lines

Upon analyzing the cellular area coverage it was observed that no significant difference was obtained between micro and macro conditions. This ensured the compatibility of microchannels as efficient cell culture platforms. However a significant higher surface area was obtained for MG-63 cells due to its fibroblastic nature as compared to the other three epithelial cell lines.

Cell specific GPMV synthesis

GPMVs were synthesized from each cell line using the earlier mentioned protocol in both macro and micro conditions. Intriguing differences were observed in the four cell line specific GPMV synthesis pattern. HaCaT cells exhibited only peripheral adjunct GPMV in micro conditions, however in macro conditions the GPMVs were freely detached and upto 20 μm in diameter. HT-29 on the other hand only supported generation of peripheral adjunct GPMV in both micro and macro conditions. MG-63 exhibited peripheral adjunct GPMV, had free detached GPMV and also greater in size. The yield was higher also due to their larger surface area.

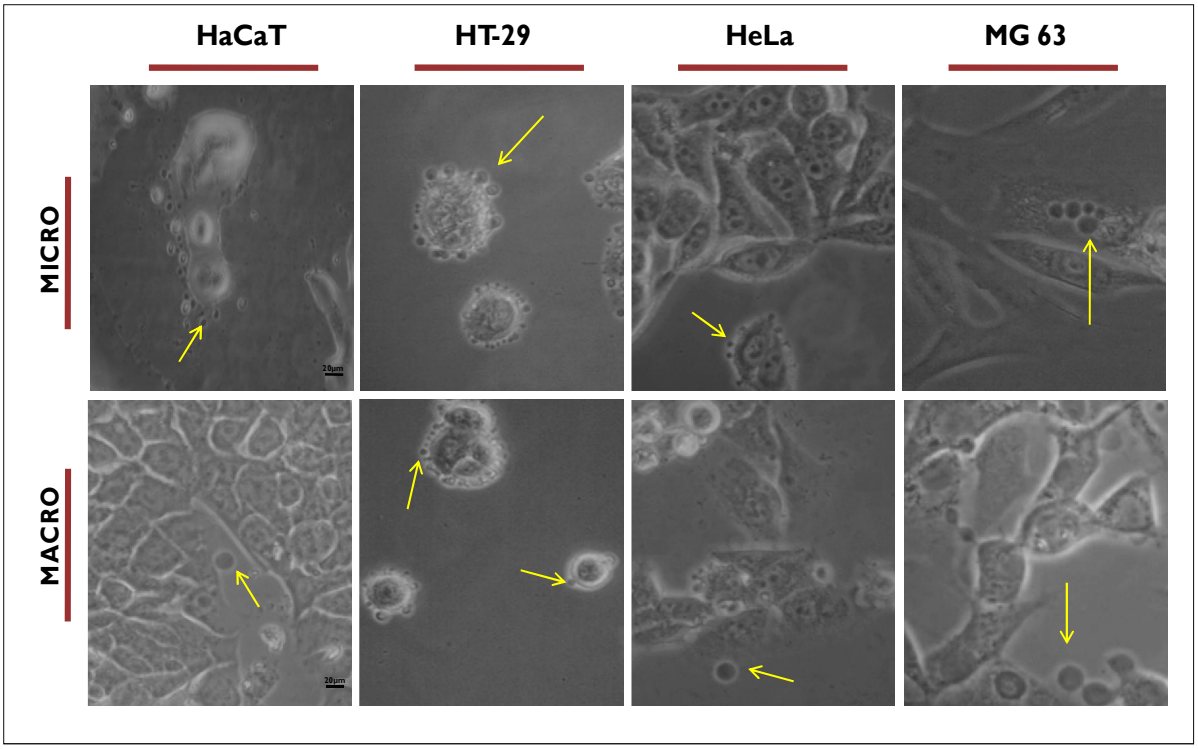

Figure 5; GPMV synthesis from four different cell lines in both micro and macro conditions

Microchannel Fabrication

Microchannels were fabricated using three different photoresist moulds, along with which inlet and outlet ports were successfully bored. Distilled water was used to test for any chokes in the microchannel due to excess PDMS application and trapped bubbles were removed prior to cell culture.. The systems were prepped for cell culture by coating under sterile conditions with 1% gelatin solution for 1 hour in 37°C.

Microchannels were successfully fabricated and found to be optimal for cell culture with varying channel height in the microscale.

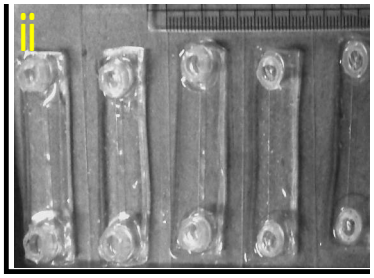

Figure 6; Schematics of the overview of microchannel fabrication procedure

SEM Analysis of Microchannel

SEM analysis was done for the cross-section of the respective microchannels in order to calculate the channel height obtained from using the three different master moulds. The channel height was found to be 104.84 ± 8.6 µm for the master mould prepared using a spin coating rpm of 1000. 2000 rpm gave rise to channel height of 72.27 ± 2.7 µm and 2500 rpm resulted in a channel height of 50.65 ± 2.0 µm

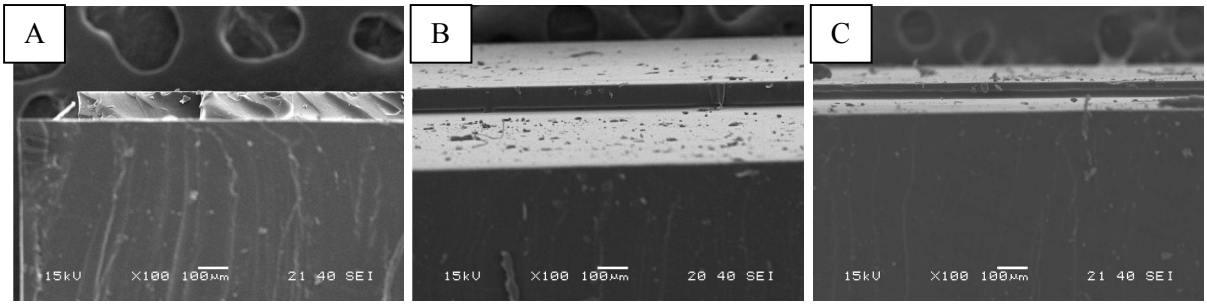

Figure 7; Cross-section of PDMS microchannels prepared by soft lithography from master moulds developed using A) 1000 rpm B) 2000 rpm and C) 2500 rpm spin coating of photoresist.

Table. 2 Showing the parametric representation of the microchannels fabricated measured using SEM analysis of the microchannel cross-section

Physical Parameters	Photoresist Application		
	1000RPM	2000RPM	2500RPM
Length (mm)	35	35	35
Width (mm)	2	2	2
Height (µm)	104.84±8.6	72.27±2.7	50.65±2.0
Volume (µL)	7.3±0.6	5.1±0.2	3.5±0.1

Channel Height dependent cell adhesion

MG-63 cells were seeded in microchannels of different height and observed under a phase contrast microscope for given time intervals to the amount of cell adhesion occurred which was calculated on the basis of cell elongation factor which is approximately 1 for cells in suspension and greater than 1 for adhered cells. With the help of this experiment the role of microconfinement was assessed since cells put under a smaller spatiotemporal arrangement adhered faster in comparison to other two microchannels.

Based on this assay the 100 µm channel height microchannel was selected to go ahead with the assay as cells in the other two channel exhibited stressed morphology. While still having the effect of microconfinemnt it tends to lie on the transition zone of culture spatiotemporal complexity.

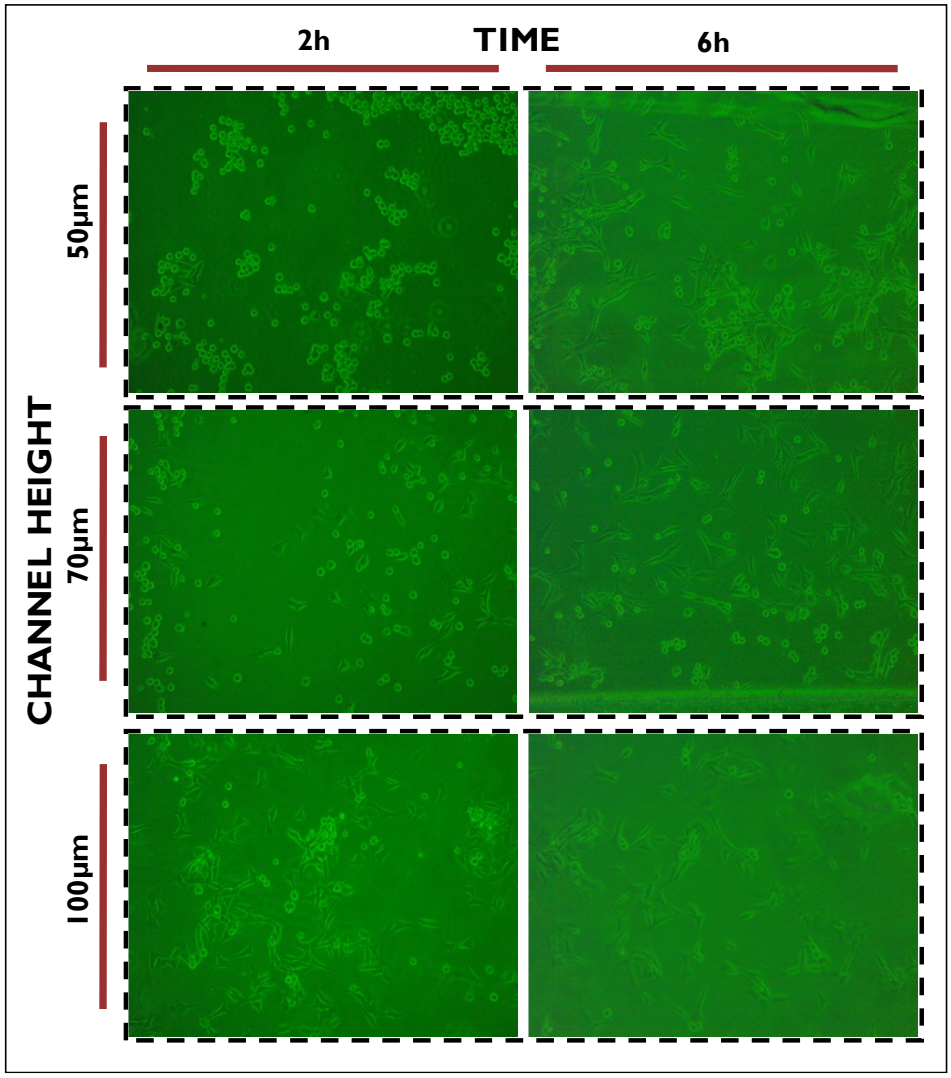

Figure 8 ; Channel **height dependent MG-63 cell adhesion**

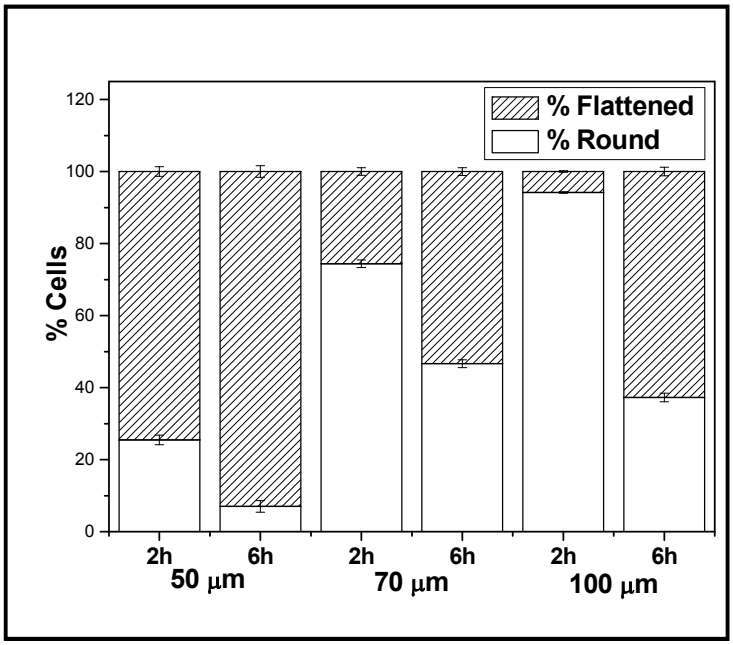

Figure 9 ; Spatiotemporal complexity of MG-63 cell adhesion in microchaanel

Channel Height dependent GPMV synthesis

Cells were cultured in microchannels of varying heights and the generation of GPMV and the role of microconfinement were assessed. It was observed that the greater the channel height the bigger the GPMV diameter however for very large microchannels the generated GPMVs tend to fuse and result in less number of GPMV per cell.

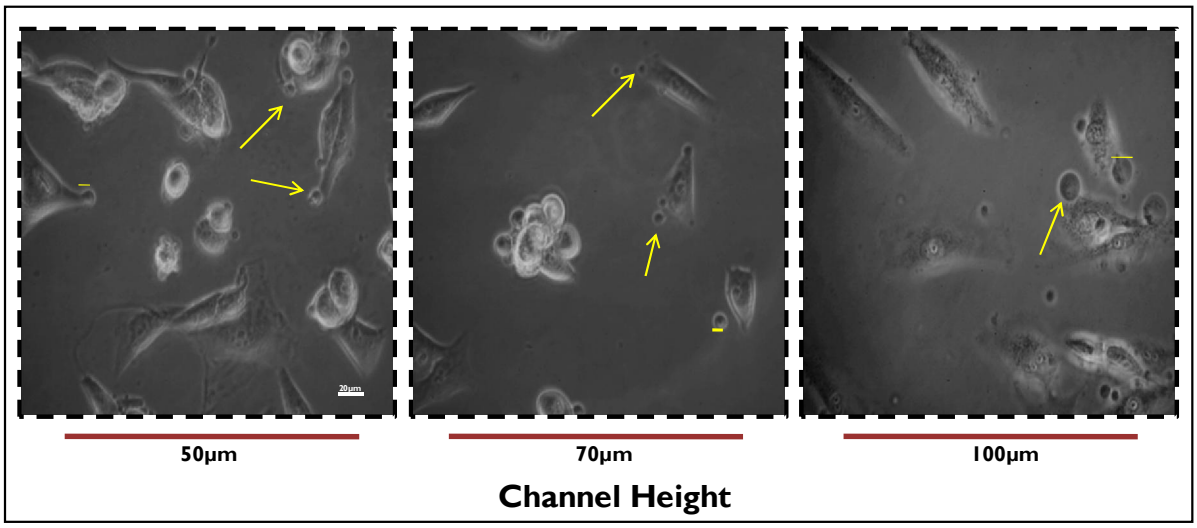

Figure 10; Channel **height dependent GPMV synthesis from MG-63 cells**

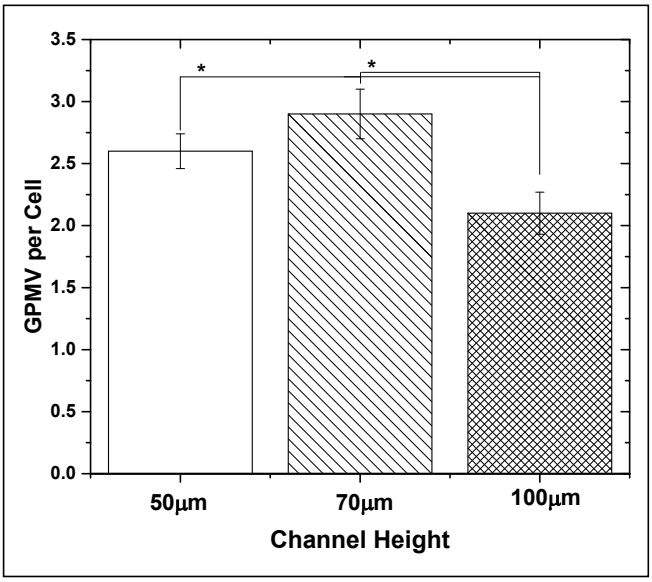

Figure 11; GPMV per cell obtained for cells grown on varying channel geight

Table.3 ; The **average GPMV diameter based on channel height significant increase**

Channel Height	Mean GPMV Diameter
50 µm	8.21±0.5 µm
70 µm	9.74±0.29 µm
100 µm	17±0.41 µm*

Fluorescence Microscopy based Growth Kinetics

Various Blockers were used to identify the membrane perturbation induced variation in GPMV synthesis. DPH anisotropy was further used to confirm the membrane fluidity and rigidity for each sample. The average GPMV diameter was also calculated for each sample.

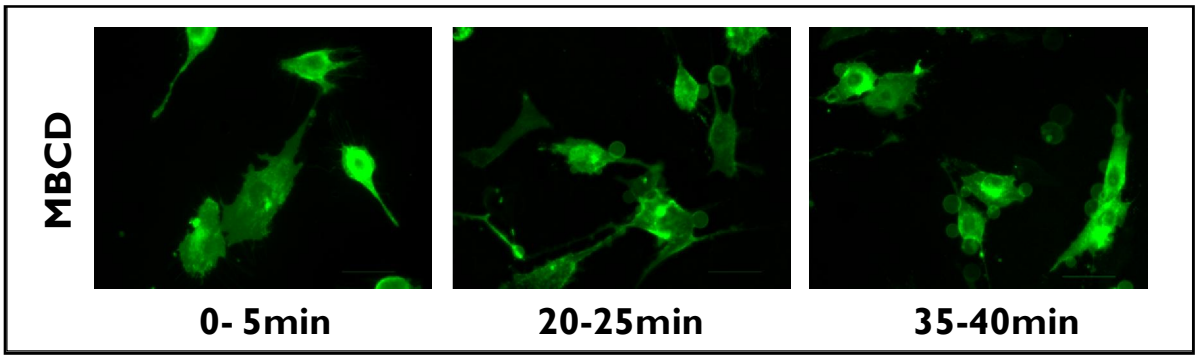

Figure 12; Time dependent GPMV synthesis upon blocker treatment

Confocal Microscopy

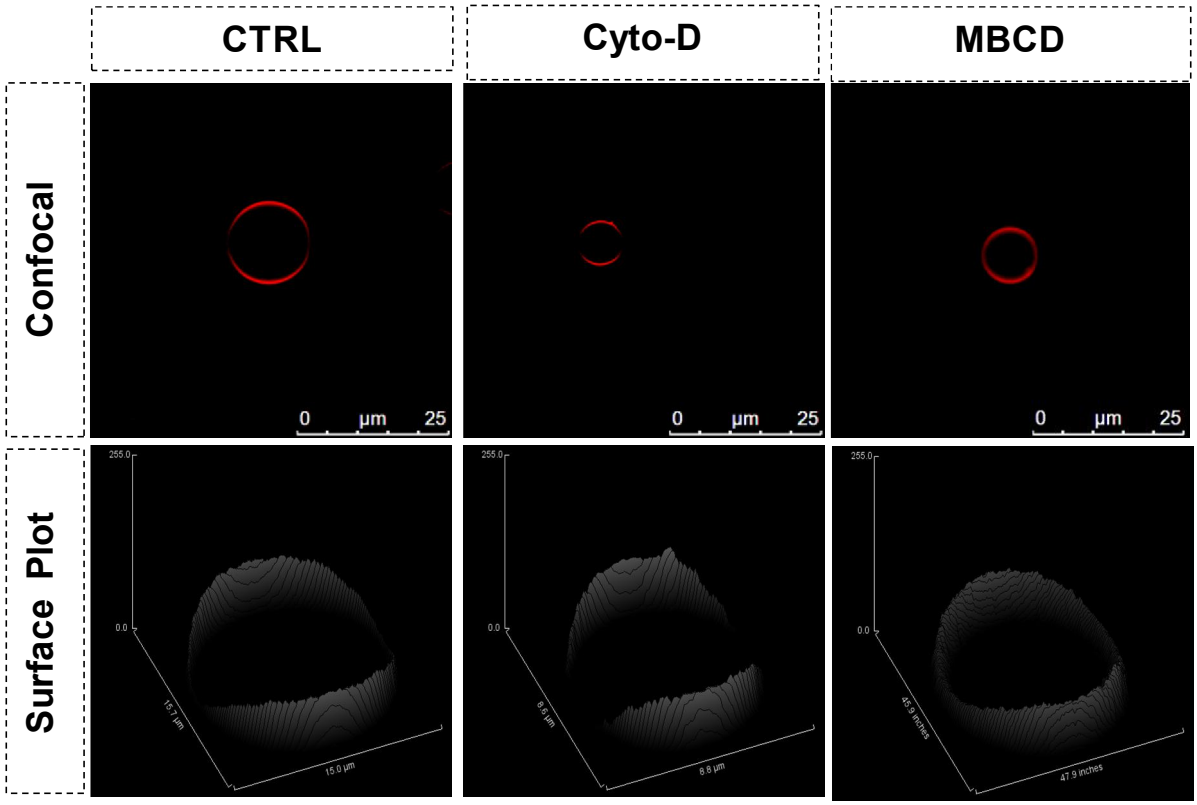

Figure 13 ; Confocal Microscopy of GPMVs

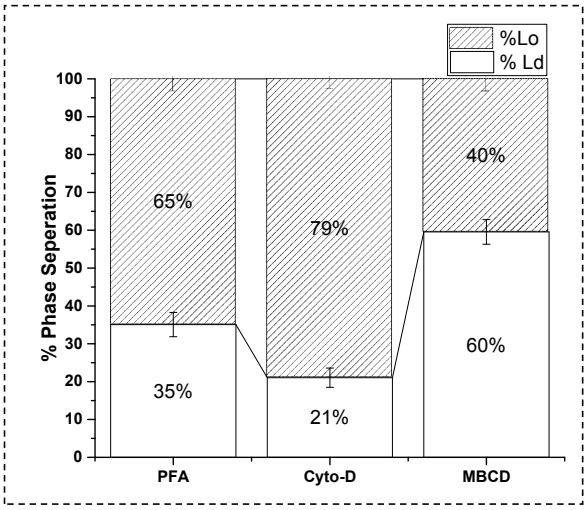

Figure 14 ; Ordered and Disordered Phase of GPMV

Confocal microscopy was carried out to identify phase partitioning of the GPMVs for the blocker treated samples. This was informative of the raft phase separation

.

Chapter 6

Conclusion

- Conventionally GPMVs have been generated with the help of chemical stressors alone but this leads to the major loss of membrane integrity and precise regulation of physical parameters is required.
- The initiative to generate vesicles with the help of physical stimuli was initiated such as use of laser and hydrostatic pressure however the limitation of loss of vesicles during purification and isolation was not circumvented.
- The ability to use microfluidics to generate size controlled vesicles due to micro-confinement.
- Subjecting cellular surface to shear stress to promote vesiculation and eventually enable use of a lower vesiculant concentration, this would enable recovery of cells post vesiculation.
- The development of a lab-on-chip model to use electrical current to isolate and collect vesicles based on their polarity would develop automated and precise vesiculation procedure.
- The vesicles can be used as membrane model; their cytoplasmic content assessed and used as therapeutic packages for targeted component delivery based on their fusion efficiency.

Scope for Further Research

Synthesizing a Lab-On-Chip device for GPMV isolation and generation on the basis of Dielectrophoresis. This would enable one manipulate the GPMVs as membrane models.

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