Synthesis and Characterization of Giant Plasma Membrane Vesicles and its Effect on Human Mesenchymal Stem Cells

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by

Deepanjali Gaur

(Roll Number: 215BM1001)

based on research carried out

under the supervision of

Prof. Indranil Banerjee

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Department of Biotechnology and Medical Engineering

National Institute of Technology Rourkela

Dept. of Biotechnology and Medical Engineering
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We the below signed, after checking the dissertation mentioned above and the official record book (s) of the student, hereby state our approval of the dissertation submitted in partial fulfilment of the requirements of the degree of *Masters of technology* in *Biomedical Engineering* at *National Institute of Technology Rourkela*. We are satisfied with the volume, quality, correctness, and originality of the work.

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May 30, 2017

Supervisor's Certificate

This is to certify that the work presented in the dissertation entitled **"*Synthesis and Characterization of Giant Plasma Membrane Vesicles and its Effect on Human Mesenchymal Stem Cells*"** submitted by **"*Deepanjali Gaur*", *Roll Number: 215BM1001,*** is a record of original work carried out by her under my guidance and supervision in the partial fulfilment of the requirements for the degree of ***Master of Technology*** in ***Biomedical Engineering*** at the ***Department of Biotechnology and Medical Engineering***, ***National Institute of Technology Rourkela***. Neither this dissertation nor any part of it has been submitted earlier for any degree or diploma to any institute or university in India or abroad.

Dr. Indranil Banerjee
Supervisor

Dedication

I dedicate this thesis to my parents and my loving sister

Declaration of Originality

I, *Deepanjali Gaur*, Roll Number *215BM1001* hereby declare that this dissertation entitled *Synthesis and Characterization of Giant Plasma Membrane Vesicles and its Effect on Human Mesenchymal Stem Cells* presents my original work carried out as a master's student of NIT Rourkela and, to the best of my knowledge, contains no material previously published or written by another person, nor any material presented by me for the award of any 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 sections "Reference" or "Bibliography". I have also submitted my original research records to the scrutiny committee for evaluation of my dissertation.

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Thinking and Feeling appreciation signifies that we believe in someone's existence rather than the divine who rules everything. This thesis entitled "**Synthesis and Characterization of Giant Plasma Membrane Vesicles and its Effect on Human Mesenchymal Stem Cells**" is the successful outcome of guidance and support bestowed on me throughout my work. I would like to express my most sincere gratitude to every single being those devoted their time for successful completion of my thesis.

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Abstract

Differentiation of mesenchymal stem cells to osteogenic lineage is a crucial step of in vivo bone remodelling and bone regeneration. In bone tissue engineering, numerous effort has been made for directed osteogenic differentiation of mesenchymal stem cells. A critical analysis of the literature revealed that in vivo, osteoblast itself induce the differentiation of mesenchymal stem cells towards osteogenic lineage. Keeping that perspective in mind here we hypothesized that giant plasma membrane vesicles (GPMV) derived from osteoblast can be as a 'packaged cues' for osteogenic differentiation of mesenchymal stem cells.

Here we have reported the synthesis of giant plasma membrane vesicles (GPMVs) from the osteoblast MG-63 using a combination of paraformaldehyde (PFA) / dithiotheritol (DTT). To analyse the effect of different cellular constituents on the physico-chemical properties of the GPMV, osteoblast cells were treated with an actin blocker (cytochalacin-D) and a cholesterol sequester (methyl-β-cyclo dextrin) prior to GPMV generation. Size, shape and structural complexicty of the GPMV was characterized by image based analysis (phase contrast microscopy, fluorescence microscopy and electron microscopy) and by flow cytometry. The lipid extracted from the GPMVs was analysed by biochemical assay and NMR spectroscopy. Further, we fused the GPMV with the hMSCs and the fusion efficiency was analysed by confocal microscopy and flow cytometry. More than 15% of Fusion efficiency was observed. Proliferation of hMSC after fusion was analysed by MTT assay, PI based live/dead assay and confocal microscopy. The osteogenic differentiation was investigated by checking the activity of alkaline phosphatase, extent of biomineralization and collagen synthesis. The RT-PCR based analysis of the expression of osteogenic markers revealed that hMSCs cultured in the presence of GPMVs shows increased expression of early and late osteogenic markers.

Key words: *Bone tissue engineering; GPMVs; hMSCs; liposome fusion; osteogenic differentiation.*

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List of Abbreviations

hMSCs:	Human mesenchymal stem cells
GPMV:	Giant plasma membrane vesicles
PMV:	Plasma membrane vesicles
PFA:	Paraformaldehyde
DTT:	Di-thiothritol
BMP:	Bone morphogenetic proteins
NEM:	N-ethylmaleimide
Cyto-D:	Cytochalasin-D
MβCD:	Methyl-β-cyclodextrin
PI:	Propium iodide
DMSO:	Dimethylsulphoxide
PBS:	Phosphate buffer saline
FBS:	Fetal bovine serum
DMEM:	Dulbecco's modified eagle media
PE:	Phycoerythrin
NMR:	Nuclear magnetic resonance
ALP:	Alkaline phosphatae
OC:	Osteocalcin
Runx2:	Runt related transcription factor
Col 1:	Collagen type 1
RT-PCR:	Reverse transcriptase - polymerase chain reaction
EDTA:	Ethylenediaminetetraacetic acid
VEGF:	Vascular endothelial growth factor
ELISA:	Enzyme-linked immunosorbent assay
DNA:	Deoxyribonucleic acid
RNA:	Ribonucleic acid
ANOVA:	Analysis of variance

Chapter 1

Introduction

Selective differentiation or site-specific differentiation of the human mesenchymal Stem Cells (hMSCs) towards osteogenic lineage is one of the pre-requisite of cell-based bone tissue engineering[1]. Many research groups have adopted different strategies to achieve the same. The methodology to achieve the same can be broadly categorised into:

1. Chemical cues

   Dexamethasone, L-ascorbic acid, bone morphogenetic proteins (BMPs) and β-glycerol phosphate are well established and extensively studied chemical factors that induces osteogenic differentiation of hMSCs [2-4].

2. Physical cues

   Mechanical factors that affect the induces osteogenic differentiation of the hMSCs are shear stress and shear strain [5].

3. Electrical cues

   Pulse-field and low frequency electromagnetic field directs the hMSCs to differentiate into osteogenic-lineage [6].

During the investigation it has been noted that in vivo, the differentiation of hMSCs got influenced by the presence of osteoblast, so far our understanding stated that it happens through the cell-cell communication as the hMSCs adjacent to the osteoblasts shows fibroblast-like morphology. The activation of the MAPKinase signalling pathway affects the transdifferentiation of the hMSCs to osteoblasts [7].

This review of the literature suggests that the osteoblast delivers some chemical signals that are responsible for the osteogenic differentiation of hMSCs. This implies that if we are able to get a package comprised of such chemical cues from osteoblast. That may be separately used for osteogenic differentiation of the hMSCs. So far, this concept has been validated in cancer research where people have proved that micro vesicles synthesized by cancer cells contain a significant amount of chemical cues for modulating the response of neighbouring cells.

In recent years, GPMVs has emerged as a promising system for studying cell membrane[8]. GPMVs are the membrane-bounded filled with fluid projections also known as blebs,

usually exclusive of the large cell organelles like golgi bodies, endoplasmic reticulum etc. which provide GPMVs a phase-lucent appearance [9]. These membrane blebs can be generated by both eukaryotic and prokaryotic living cells. The vesiculating agents plays an important role in the synthesis of the membrane blebs as the paraformaldehyde causes non-specific cross-linking of the proteins and di-thiothritol causes cleaving of the disulphide and thioester bonds. The membrane actin known as cortical actin is present to provide structural stability to the system, naturally when the actin present cannot retain the cytosolic pressure to maintain the stability of the cell morphology it releases the membrane blebs to regain its cellular stability and structure [10].

So far it has been observed that the GPMVs contain some information. Keeping this perspective in mind, here we have hypothesized that GPMV generated from osteoblast may have the capability to induce osteogenic differentiation of hMSCs.

Under the stress-inducing conditions the blebbing of the membrane is observed and it's an indication of cell-injury but the blebs formed are reversible blebs. These blebs have been explored and analysed thoroughly. On the other hand, the mechanism of generation and characterization of GPMVs is still not completely understood. Over the years, GPMVs have been proven an excellent acellular system to investigate the complexity and composition of the cell membrane. As the GPMVs are generated from live cells the native form of the cell membrane is conserved [11].

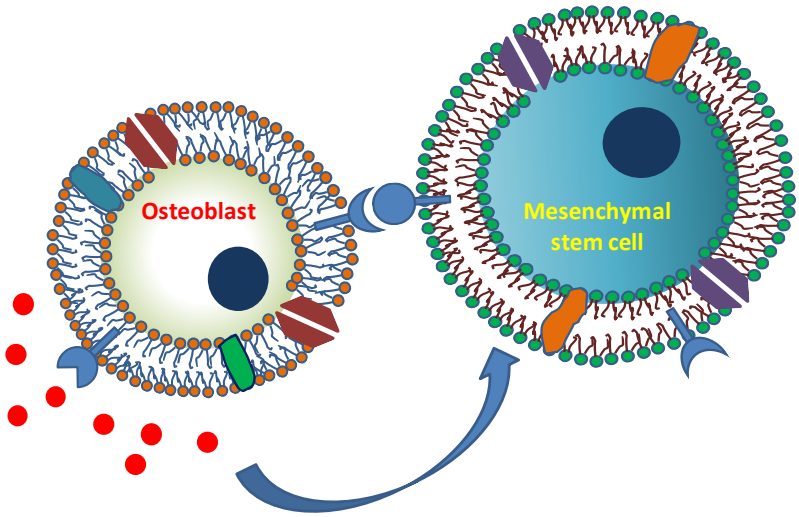

Figure 1.1: Schematic representation of the in vivo bone remodeling by juxtacrine and paracrine signaling

Chapter 2
Literature Review

2.1 Plasma Membrane Vesiculation

Studying the composition and complexity of the cell membrane has always been an interesting topic among the researchers as it determines and gives better understanding of the in vivo signalling and other cell membrane mediated pathways. But the cell membrane system is tough to handle in the native structure. So, it leads to the development of new techniques to study the cell membrane system. Generation of membrane blebs is one such method of extracting the cell membrane in its native form and study it. Plasma membrane vesicles have proven to be an excellent model system. Chemical factors known as vesiculating agents have been extensively studied for the synthesis of membrane blebs. N-ethymaleimide (NEM), paraformaldehyde(PFA) and di-thiothritol (DTT) [14] are currently used to induce the vesiculation of the cell membrane. Membrane vesicle can be used as the excellent delivery vehicle to drugs, growth factors and proteins. As derived from the the living system it maintains the natural composition and complexity of the cell membrane from which it has been synthesized.

2.2 Giant Plasma Membrane Vesicles

In 1919 Mary Jane Hogue reported the chemicals that can be used as the generate blebbing of the cell membrane. The hypertonic and hypotonic solution of various salts can be used to achieve the blebbing of the cells [15]. The older techniques to generate the were to homogenize the cells, perform isopycnic centrifugation etc. In 1976 Barry L Jacobs *et.al* reported a new method of generating the vesicles from the plasma membrane which can be further used to study the model system. The formaldehyde at a concentration of 25 mM to 250 mM, 10 mM of N-ethymaleimide in the PBS buffer can generate the plasma membrane vesicles (PMVs), Di-thiothritol along with formaldehyde working solution can be used [16]. Giant plasma membrane vesicles (GPMVs) are the vesicles generated from the plasma membrane of the desired cell line. Various alkylating-cysteine chemical treatment induces the generation of the GPMVs.

2.3 Blockers Treatment

Various blockers like colchicine and cytochalasin-b were used to block the generation of GPMVs [17], keeping the cells at 4ºC stops the generation of the plasma membrane vesicles. Here we have chosen two different blockers to study their effect on the synthesis and composition of the GPMVs.

2.3.1 Cytochalasin-D

The cytochalasin-D has been proven that it inhibits the polymerization of the actin monomers by inducing the secondary conformational changes in monomers in the presence of Mg^{+2}. The actin plays an important role in the generation of the GPMVs as the blebbing of the membrane occurs when the cortical actin maintaining the cytoskeleton of cell can no longer retain the intracellular pressure. So, we want to investigate the effect of actin polymerization blocker on the kinetics of GPMVs generation and composition of lipids and proteins present in the generated GPMVs as compared to the control.

2.3.2 Methy-β-Cyclodextrin

One of the significant part of the eukaryotic cell membrane, it provides the fluidity to the cell membrane. Methy-β-cyclodextrin (MβCD) is the cholesterol sequester, depleting the cholesterol from the cell membrane by forming a MβCD-cholesterol dimer complex. MβCD can limit the growth kinetics of the cell by altering the composition of the plasma membrane [18].

2.4 Bone Tissue Engineering

Due to increased occurrences of bone disorders, the need for bone grafts is increasing corresponding to the demand. But, the availability of the autografts and allografts depends on the various factors which cannot be controlled. After several years of research still, the autograft serves as the gold standard as it does not face graft rejection from the host's body, the biggest disadvantages are double surgery and highly expensive. Allografts are highly histocompatible they can also be used after decellularization of bone matrix, bone chips etc.[19]. The various other strategies adopted to repair the bone disorders or fractures include bone cement and involvement of bone morphogenetic proteins. The bone graft designed in vitro should be osteoinductive as well as angiogenic in nature to support the regeneration of the bone on the site of implantation. This leads to the necessity to develop

the bone grafts in vitro for implantation into the patients.

2.5 Human Mesenchymal Stem Cells

Human mesenchymal stem cells (hMSCs) are the undifferentiated multipotent stem cells capable of differentiating mainly into different tissue types of mesenchymal lineages for example mesenchymal tissues such as bone, cartilage, muscle, ligament, tendon, adipose, and stroma and different cell types like fibroblast, osteoblast, adipocyte and chondrocytes. hMSCs are uniformly positive for these surface markers SH2, SH3, CD29, CD44, CD71, CD90, CD106, CD120a, CD124 and HLA-DR [20]. For osteogenic differentiation various chemical inducer are used like dexamethasone, β-glycerol phosphate, and ascorbate was prepared in the complete hMSCs expansion medium. For adipogenic differentiation was induced by treating the cells with 1-methyl-3-isobutylxanthine, dexamethasone, insulin, and indomethacin in complete expansion medium [21]. To induce the chondrogenic differentiation of the mesenchymal stem cells the isolated cells were cultured with transforming growth factor–b3 in incomplete expansion medium.

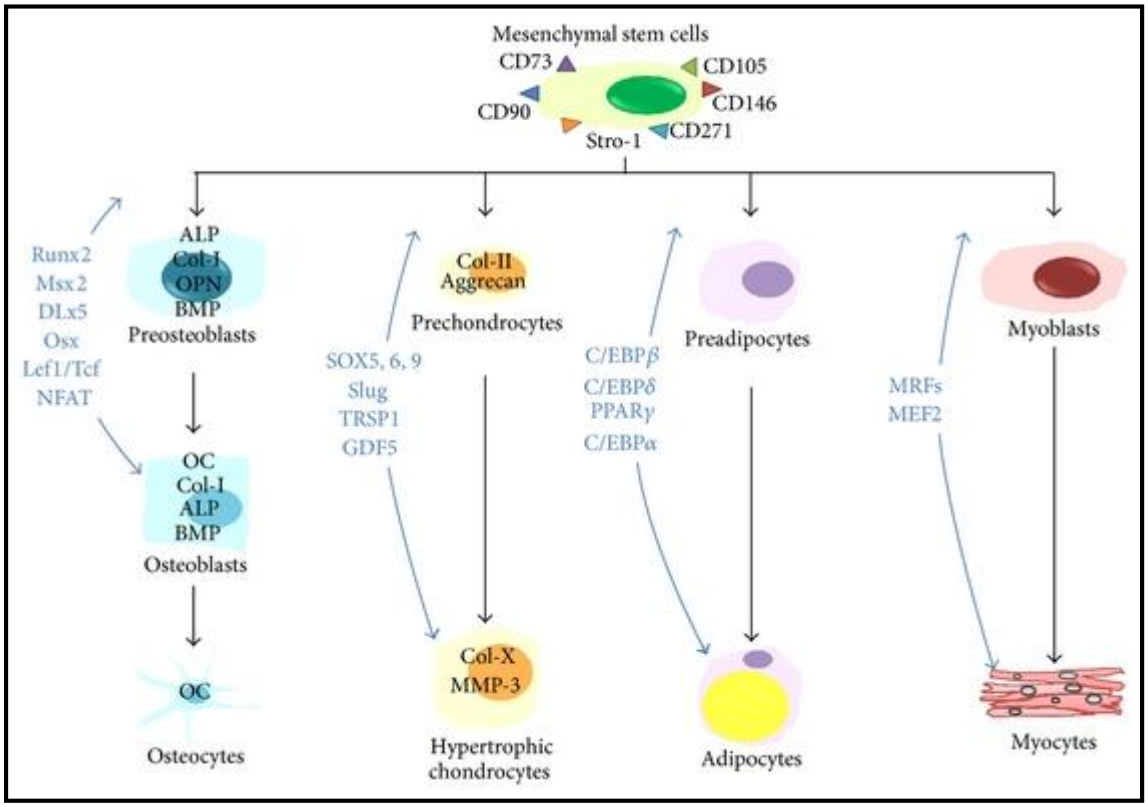

Figure 2.1: Differentiation lineages of human Mesenchymal Stem Cells (21)

2.6 Strategies for inducing Osteogenic differentiation of hMSCs in vitro

The in vivo bone remodelling and regeneration takes place due to the induction of the hMSCs into osteogenic lineage by the neighbouring osteoblast. So, researcher have achieved the same in vitro by co-culture of the osteoblast with the hMSCs. One of the major disadvantage of the co-culture is the different growth medium requirement by the different cell lines [21]. The induction of osteogenic differentiation by using the chemical factors is promising methodology. The mechanical factors like shear stress and shear strain has also proven to induce the osteogenic differentiation of the stem cell. Low frequency pulsed field electromagnetic induction also induces the same.

2.7 Liposome Fusion Efficiency

The fusion of the membrane is a naturally occurring process in the living organism to perform different tasks like repairing the damaged organelles, signalling pathway in the extracellular matrix, cell-cell communication and for the cells to adhere to the developing tissue or organ. These behavioural characteristics can be further investigated and explored for various biomedical and biotechnical applications. Micro-injection, treatment with trypsin, polyethylene glycol [22] are few chemical inducers that promotes the fusion of the liposome with liposome or liposome to cell membrane.

Chapter 3

Objectives and Work Plan

3.1 Objectives

1. Synthesis and characterization of giant plasma membrane vesicles from osteoblasts.
2. Analysis of the fusion efficiency of giant plasma membrane vesicles with the human mesenchymal stem cells.
3. Investigation of the osteogenic differentiation of human mesenchymal stem cells cultured in the presence of giant plasma membrane vesicles.

3.2 Work Plan

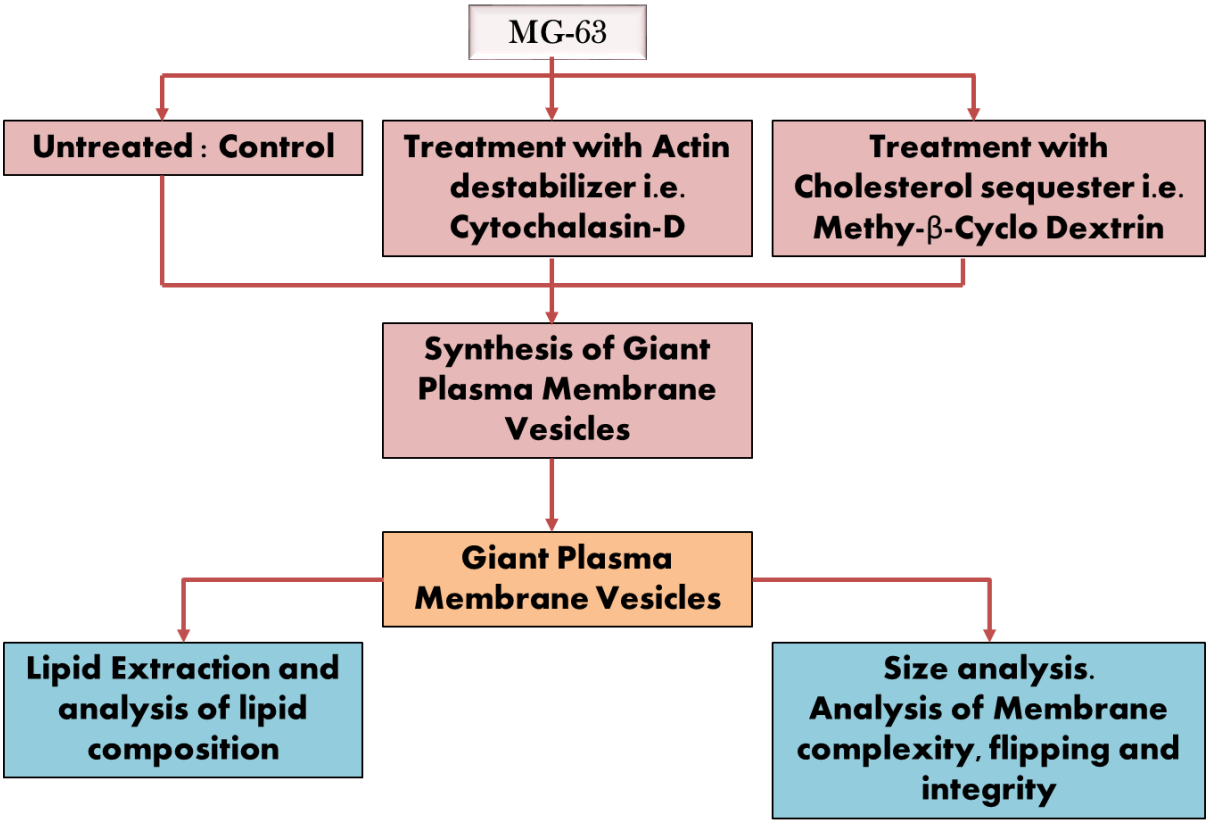

Figure 3.1: Schematic representation of the work plan for objective one

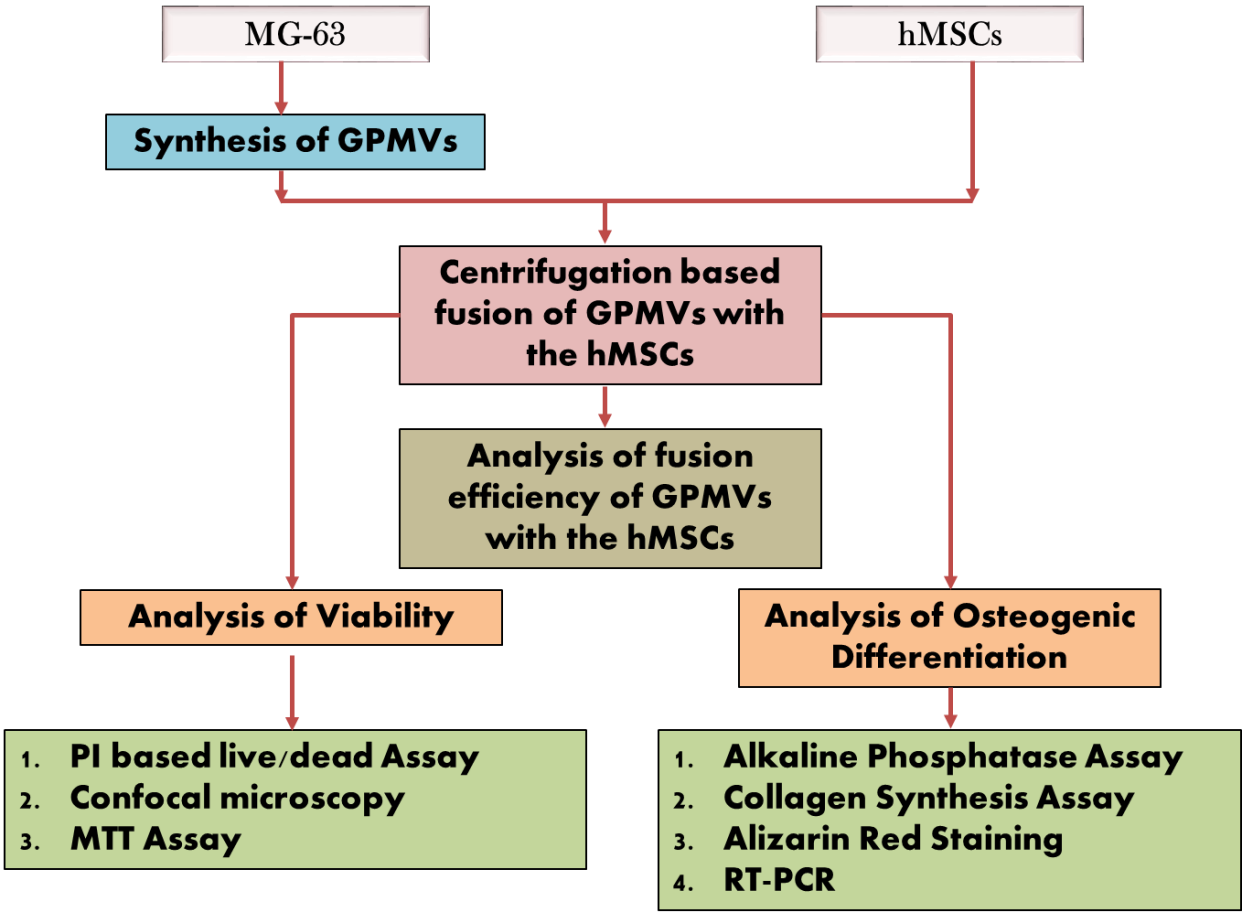

Figure 3.2: Schematic representation of the work plan for objective two and three

Chapter 4

Material and Methods

4.1 Materials

The cell line MG-63 was obtained from NCCS, Pune. Human mesenchymal stem cells were obtained from HIMEDIA. India.Dulbecco's Modified Eagle Media (DMEM), Fetal bovine serum (FBS), Dulbecco's Phosphate buffer saline (DPBS), Antibiotic-antimycotic solution, trypsin-EDTA, paraformaldehyde, MTT assay kit, di-thiothreitol (DTT), glutaraldehyde, dimethylsulphoxide (DMSO), alizarin red, dexamethasone, L-ascorbic acid, β-glycerol phosphate was purchased from HIMEDIA. Ethanol was purchased from Merck. Propidium iodide (PI) was obtained from Sigma-Aldrich, Mumbai, India. Vybrant Dio, Cell Tracker Red and PI/RNase solution were obtained from Invitrogen, USA. Syringe water was purchased from Apollo pharmacy, NIT Rourkela, Orissa, India. Autoclaved distilled water was used throughout the experiments.

4.2 Methodology

4.2.1 Synthesis of Giant Plasma Membrane Vesicles

For GPMV synthesis, osteoblasts (MG-63) were harvested by trypsinization and the cells were seeded at a density of $5.0* 10^5$ cells per ml into T-25 culture flask. After two days of incubation, cells were washed once with GPMV buffer (10 mM HEPES, 150 mM NaCl, 2mM CaCl2, pH 7.4), 1.5 ml of GPMV reagent was added (25 mM formaldehyde and 3 mM DTT in GPMV buffer) and observed under the phase contrast microscope (Carl Zeiss Primovert). After 1 h of incubation at 37°C, the supernatant containing the GPMVs was taken and stored at 4°C until further use [10].

4.2.2 Labelling of Giant Plasma Membrane Vesicles

For labelling the GPMV, the MG-63 cells were stained with the DiO working Solution (1µl vybrant DiO in 1 ml of Complete media) for 20 min. Wash once with GPMV buffer and then follow the aforementioned protocol to synthesise GPMV. After the overnight incubation, the GPMV suspension was centrifuged at 8000 rpm for 10 min. The 500µl of the GPMVs solution was discarded and a residual suspension containing GPMV was observed under Lieca SP8 confocal microscope [10].

4.2.3 Synthesis of GPMV in presence of blockers

For GPMV synthesis after the blocker treatment, two 70% confluent T-25 culture flask of MG-63 was taken, one was treated with cytochalasin-D an actin polymerization blocker it will inhibit the actin monomers to form the polymer and disrupts the cytoskeleton of the cells and the other one with methyl-β-cyclodextrin (MβCD) a cholesterol depleting agent it forms dimer complex with the cholesterol, once the cholesterol has been depleted the membrane fluidity will decrease providing rigidity to the cells. Cells were washed twice with PBS before blocker treatment. Blocking solution was added to culture flask. Working concentration of 10µM was used for cytochalasin-D and the time of exposure was optimized to be 30 min. and 500 µM was used for MβCD and the time of exposure was optimized to be 30 min. The MG-63 Cells were treated with the blockers and observed under Carl Zeiss Primovert Phase Contrast Microscope.

4.2.4 Size analysis of Giant Plasma Membrane Vesicles

4.2.4.1 Phase Contrast Microscopy

After adding the GPMV buffer to the control and blocker treated flasks. The flasks were observed at different time period. At 400 X magnification to analyse the time dependent effect of GPMV buffer on cells and kinematics of the cells and investigate the effect of the blockers on the size and kinematics of the cells after the treatment with blockers i.e. cytochalasin-D and methyl-β-cyclodextrin.

4.2.4.2 Environmental Scanning Electron Microscopy (ESEM)

After collecting the supernatant from the T-25 culture flask incubated with GPMV buffer for the generation of GPMV from the osteoblast cell line i.e. MG-63. The GPMV suspension was stored at 4ºC overnight. After overnight incubation the GPMV suspension was centrifuged at 8000 rpm for 10 min. 500µl from the supernatant was removed and from the remaining GPMV suspension, 10µl of the supernatant was taken and drop was dried on glass slide overnight. The sample was observed under ESEM at 6000 X magnification.

4.2.4.3 Fluorescence Microscopy

Prior to the GPMV generation, the cells were stained with the DiO staining solution using the protocol mentioned in section 4.2.2. The cells were washed once with PBS and then working solution of the DiO was added and incubated for 20 min. The cells were treated prior to the generation of the GPMV the mg-63 cells were treated with cytochalsin-D and

methyl-β-cyclodextrin. Incubated for 30 minutes and then working solution of the was removed and washed twice with the PBS and GPMV buffer was added and observed under the fluorescence microscope for three different time-intervals i.e. 0-10 min., 20-25 min. and 40-50 min.

4.2.5 Analysis of membrane flipping of the GPMVs by Annexin V

4.2.5.1 Samples

Before generating the GPMVs the cells the cells were stained with DiO as aforementioned protocol. The blocker was done using the protocol mentioned in the section 4.2.3. The 2% annexin-V solution was prepared in the GPMV working solution so that the membrane flipping can be observed and phosphatidylserine flipping is present the blebs formed, and are the blebs different from the apoptotic blebs. The cells were incubated for 20 minutes in the working solution and then fixed using the 4% paraformaldehyde solution to observed the flipping in the membrane while the GPMV are in due process of generation and then observed under the lieca SP8 Confocal microscope and the image analysis was done using the leica lasX software.

4.2.5.2 Positive Control

For the positive control meaning to induce the apoptosis in the cells. The cells were treated with 125 µM solution of hydrogen peroxide which is an apoptotic inducer. Cells were incubated with hydrogen peroxide for 2 hr. and then washed once with the PBS and then 2% annexin-V solution was added. The cells were incubated for 20 minutes in the working solution of hydrogen peroxide and then fixed using the 4% paraformaldehyde solution The cells were observed under the Lieca SP8 confocal microscope and the image analysis was done using the Leica lasX software.

4.2.6 Analysis of membrane integrity by Propidium Iodide

After the GPMV was generated as aforementioned protocol the GPMV was stored at 4ºC overnight. After overnight incubation, the GPMV were centrifuged at 8000 rpm for 10 min. Upper half volume of the buffer was removed and propidium iodide working solution was added to the GPMVs and incubated for 20 min. to analyse the membrane integrity of the cells as the propidium iodide will bind to the DNA but it can only penetrate inside the cells if the membrane is disintegrated. The flow cytometric analysis of the samples was done using the C6 BD accuri flow cytometer and data was analysed using C6 BD accuri software.

4.2.7 Analysis of membrane Complexity by Flow Cytometry

The cells were stained with DiO using the protocol mentioned in section 4.2.2. prior to the GPMV generation as aforementioned protocol. The cells were treated with the blockers before generating the GPMVs. The GPMVs using the PFA/DTT working solution for all the three samples i.e. for the control and blocker treated cells and analysed using the C6 BD accuri flow cytometer and data was analysed using C6 BD accuri software for side scatter and forward scatter as the side scatter will define the complexity of the membrane and forward scatter will give the information about the size of the sample.

4.2.8 Lipid extraction from GPMV

The lipid analysis of the lipid extracted from the GPMV generated from the untreated and treated MG-63. To analyse the composition of the lipid the lipid was extracted using the protocol given by Dyer and Bligh after doing the necessary modification in the protocol followed. The GPMVs were generated using the protocol mentioned in the section 4.2.1.the GPMV suspension was kept for overnight incubation at 4ºC. lipid extraction was performed using the methanol: chloroform ratio of 2: 1. 2 ml of chloroform: methanol solution was added to 2 ml of GPMV suspension. The suspension was mixed rigorously and left untouched for 30 min. for the phase separation of the two layers. The bottom layer was collected in the separate tube. The lipid extraction process was repeated twice using the chloroform and GPMV suspension in the ration of 1: 1. The bottom layer was collected in the same tube. After the lipid extraction the solution was washed once with the 1 M KCl solution for the removal of the salts. The KCl solution and lipid solution was added in the ratio of 0.4:1 the solution was shaken rigorously and left untouched for 30 min. after the incubation the upper layer was removed. To remove the chloroform and obtain the lipid the samples were dried using a rotating evaporator. The extracted lipid was stored at room temperature until further downstream analysis.

4.2.9 Characterization of the Lipids Extracted from the Giant Plasma Membrane Vesicles

4.2.9.1 Biochemical Assay

4.2.9.1.1 Cholesterol Estimation Assay

The quantification of the cholesterol was done using the protocol given by Bowman and Wolf by modifying the protocol to perform the assay. The solution of ferric chloride was

prepared by taking 0.8 ml of 2.5% solution of ferric chloride. The ferric chloride was prepared in the glacial acetic acid. The water was added to prepare 85% of H_3PO_4 was prepared using the 9.2 ml of concentrated sulphuric acid. The lipid was extracted from the generated GPMV using the chloroform methanol extraction process. The samples were dried in the inert nitrogen system. The dries samples were diluted using the 500µl of the ethanol and 500 µl of the ferric chloride solution. The solution was incubated for 30 minutes, pinkish colour shows the presence of the cholesterol. and then the absorbance was taken at 550 nm.

4.2.9.1.2 Phosphorus Estimation Assay

To evaluate the amount of total phosphorus present in the lipid extracted by using chloroform methanol extraction process, as the total phosphorus will directly corresponds to the total phospholipid present in the system. Potassium dihydrogen phosphate was taken as the standard for the quantification. The standard samples were prepared in water from 0.01µM to 0.04 µM concentration was used. The dried lipid samples were taken and 90µl of 8.9 N Sulphuric acid was added. After the addition of sulphuric acid tubes were heated at the temperature of 210ºC for 25 min. after the heating leave the test tubes to cool down. After the tubes have cooled down add 30µl of hydrogen peroxide and heat for 30 min. at 210 ºC. let the test tubes cool down and add 780µl of water. Add 100µl of ammonium molybdate tetrahydrate solution and solution was mixed thoroughly using vortex and 10µl of ascorbic acid was added. The test tubes were heated at 100º for 7 min. absorbance was taken at 820 nm using UV-Visible spectrophotometer. The standard curve was plotted to determine the concentration of phosphorus present in the samples.

4.2.9.2 Nuclear Magnetic Resonance (NMR) spectroscopy

The lipids were extracted by aforementioned protocol using chloroform methanol extraction process and dissolved in the 2.5ml of $CDCL_3$: $CD_3O.D.$ (2:1) and 500µl of sample in the NMR tube using the syringe and analyse for proton and phosphorus spectroscopy and analysis by GSim NMR spectroscopy software [23].

4.2.10 Labelling of hMSCs with Cell Tracker (Red)

T-25 culture flask of hMSCs was taken at 60-80% confluency, spent media was removed. The cells were harvested by trypsinization. 1 ml of cell tracker (1X) was added to the cell suspension and incubated for 45 min. at 37°C. After the incubation, the cell suspension was

centrifuged. The supernatant was removed and the cells were washed twice with media. The cell pellet was obtained by centrifugation and re-suspended in 1ml of media until further use.

4.2.11 Fusion of DiO labelled GPMV generated from MG-63 to cell tracker labelled hMSCs

500µL of the cell suspension was taken and 500µL of the GPMV was taken after overnight incubation at 4ºC and then the suspension was centrifuged 12000 rpm for 10 minutes and the upper supernatant was removed and then lower supernatant was taken in the microcentrifuge tube along with the cell suspension and was centrifuged at 2000 rpm for 2 min. The ratio of cell: GPMV was kept 10:1 to increase the fusion efficiency of the GPMVs with the cells. The cells were seeded accordingly for further downstream analysis.

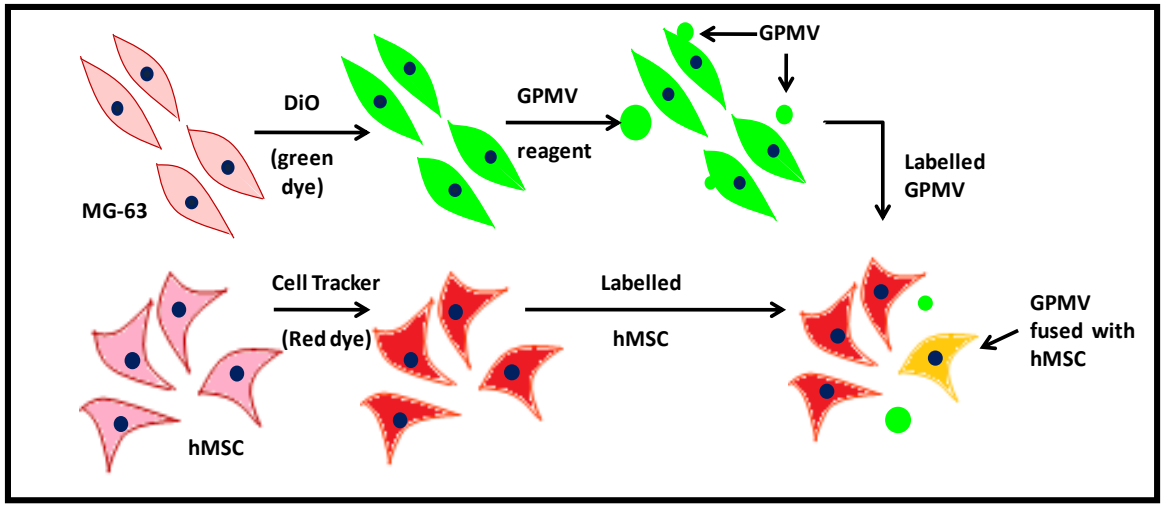

Figure 4.1: Schematic representation of the fusion of GPMV with the human mesenchymal stem cells.

4.2.12 Analysis of the Fusion efficiency of hMSCs with GPMV

4.2.12.1 Confocal microscopy

The hMSCs were centrifuged with the GPMV and after that seeded in the prepared confocal wells sticking the wells with PDMS on the glass cover slips and incubated for 2 h and then after fixed with 4% paraformaldehyde solution and then observed under leica TCS SP8 confocal microscope and was analysed using leica LAS-X software.

4.2.12.2 Flow Cytometry

The hMSCs were centrifuged with the GPMVs (stained with the DiO) to induce the fusion and the flow cytometric analysis was done to analyse and verify the fusion efficiency of the

cells with the GPMV using the BD C6 flow cytometer and the analysis was done using the FCS express software.

4.2.13 Cell proliferation study of the hMSCs Cultured in presence of GPMV

4.2.13.1 Phase Contrast Microscopy

The hMSCs were centrifuged with the GPMV and after that seeded in the 12-well culture plate and incubated for 24 h and 96 h and observed under carl zeiss phase contrast microscope at 100X magnification.

4.2.13.2 Confocal Microscopy

The hMSCs were centrifuged with the GPMV (stained with Dio dye using the protocol mentioned in the section 4.2.2.) and after that seeded in the confocal wells and incubated for 12 h were observed under leica TCS SP8 confocal microscope and was analysed using leica LAS-X software.

4.2.13.3 MTT assay

hMSCs with GPMV were seeded at the density of $5* 10^3$ cells/well in 96-well culture plate and incubated for 24 h and 96 h. Cell proliferation was assessed by MTT assay to investigate the effect of GPMV and GPMV buffer on the morphology and growth of the hMSCs. The absorbance was measured at 595nm.

4.2.13.4 Propidium Iodide(PI)-Based live/dead assay

The hMSCs with GPMV were seeded at a population of $1* 10^5$ cells/well in 6-well culture plate and incubated for 24 h and 96 h. The Cells were harvested using the trypsin-EDTA and the cell suspension was centrifuged and the supernatant was removed. The cells were re-suspended in the media containing the propidium iodide working solution to the stain with the PI and incubated for 20 min. at room temperature in dark. Vortex each sample before analysing it to make single cell suspension. The samples were analysed using C6 BD accuri flow cytometer and data was analysed using C6 BD accuri software.

4.2.14 Preparation of differentiation media

The differentiation media was prepared using 100nM of dexamethasone, 10nM of L-ascorbic acid and 30nM of β-glycerphosphate in the low glucose expansion media containing 10 % FBS and 1% antibiotic-antimycotic solution.

4.2.15 Osteogenic differentiation study of the hMSCs cultured in presence of GPMV

The cells were seeded at the density of 5* 10^4 cells/well in 12-well culture plate after the fusion was performed using the aforementioned protocol. Following assays was performed as mentioned in the protocols below:

4.2.14.1 Phase Contrast microscopy

The hMSCs were centrifuged with the GPMV and after that seeded in the 12-well culture plate and incubated for 14 days and observed under carl zeiss phase contrast microscope at 100X magnification.

4.2.14.2 Alkaline phosphatase Assay

After 14 days of incubation, the cells were trypsinized using trypsin-EDTA, the cell was lysed using 0.1% of triton-X. 50µl of cell lysate was taken and 50µl of alkaline phosphatase buffer was taken, the plate was incubated overnight and taken the O.D. at 405 nm.

4.2.14.3 Alizarin Red staining

After 14 days of incubation, cells were washed once with PBS, 1 ml of dye was added for 1 h. The plate was kept on an orbital shaker. The supernatant was removed and washing was done thrice to remove the dye with water, the plate was kept on an orbital shaker. Observed under the carl zeiss primovert phase contrast microscope. 10% cetyl pyrimidine chloride was added and incubated for 30 minutes. Absorbance was taken at 550 nm.

4.2.14.4 Collagen estimation Assay

After 14 days of incubation, cells were washed once with PBS**.** The cells were fixed using the bouin's fluid for 1 h under continuous shaking. Fixing solution was removed and the sample was air dried. Sirius dye solution was added and the plate was incubated for 1 h under continuous shaking. The dye was removed and cells were washed once with HCl (0.01N) solution and observed under carl zeiss primovert phase contrast microscope. After microscopic analysis, NaOH (0.1N) solution was added and was kept on a shaker for 30 min. Absorbance was measured at 550 nm.

4.2.14.5 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

The mRNA was isolated and synthesized using the one-step Qiagen RNeasy Kit and then then cDNA was synthesized using the Bio BhartiMuLV Reverse Transcriptase Kit and the

steps were followed as written in the user manual. The genes were amplified using the primers mentioned in the table below and running the PCR for 25 cycles. The samples were analysed by running the samples on the 8% agarose gel containing 1% of ethidium bromide solution. The band intensity analysis was done by using the NIH Image J software. Glyceraldehyde 3 phosphate dehydrogenase was taken as control for the analysis.

Table 4.1: List of Primers used for osteogenic differentiation study of the hMSCs fused and cultured in the presence of GPMV

Gene	Primer Type	Primer sequence (5'-3')
Glyceraldehyde 3 phosphate dehydrogenase (GAPDH)	Forward	CATGAGAAGTATGACAACAGCCT
	Reverse	AGTCCTTCCACGATACCAAAGT
Runt-related transcription factor 2 (RunX2)	Forward	TTTACRRACACCCCGCCAGTC
	Reverse	CAGCGTCAACACCATCATTCTG
Alkaline Phosphatase(ALP)	Forward	GACAAGAAGCCCTTCACTGCC
	Reverse	CAGACTGCGCCTGGTAGTTGT
Collagen type I (Col1)	Forward	AGACTGGCAACCTCAAGAAGGC
	Reverse	CGGGAGGTCTTGGTGGTTTTGT
Osteocalcin (OC)	Forward	GTGCAGAGTCCAGCAAAGGTG
	Reverse	TCAGCCAACTCGTCACAGTCC

4.1.16 Osteogenic differentiation study of the hMSCs cultured in presence of GPMV buffer

The cells were seeded at the cell density of 5* 10^3 cells per well in 12-well culture plate with three different concentrations of the GPMV buffer i.e. 50µl, 100µl and 200µl. Following assay was performed as mentioned:

Alkaline phosphatase Assay

After 14 days of incubation, the cells were trypsinized using trypsin-EDTA, the cell was lysed using 0.1% of Triton-X. 50µl of cell lysate was taken and 50µl of alkaline phosphatase buffer was taken, the plate was incubated overnight and taken the O.D. at 405 nm.

4.1.17 VEGF expression study of the hMSCs cultured in presence of GPMV

100µl of the sample was taken from the growth medium of the cells and added to the pre-coated wells and incubate for 2h. on orbital shaker. After the incubation the wells were washed for 6 times with the wash buffer The 100µl of biotin-conjugated primary antibody was added and incubate for 1 h. on orbital shaker. The wells were washed properly with the wash buffer. 100µl streptavidin-HRP was added to the plates and incubated for 30 min. in dark. After 30 min. of incubation add 100µl of STOP solution and the absorbance was observed at 620 nm using ELISA microplate reader.

4.1.18 Statistical analysis

All the data were analysed for statistical significant difference between the samples using the ANOVA-single factor method of analysis, where $\alpha=0.5$ and p-value < 0.05.

Chapter 5

Results and Discussion

5.1 Synthesis of Giant Plasma Membrane Vesicles

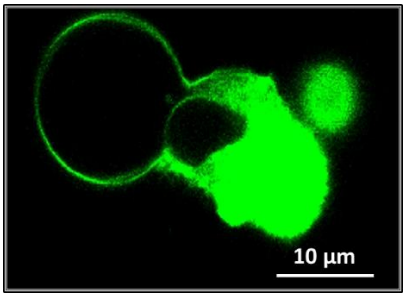

Figure 5.1: Synthesis of GPMVs stained with vybrant DiO and observed under confocal microscope

The labelling of the MG-63 cells with vybrant Dio was properly done, as we can observe in figure 5.1 that the synthesis and labelling of the GPMV is a simultaneous process.

5.2 Size analysis of Giant Plasma Membrane Vesicles

5.2.1 Phase Contrast Microscopy

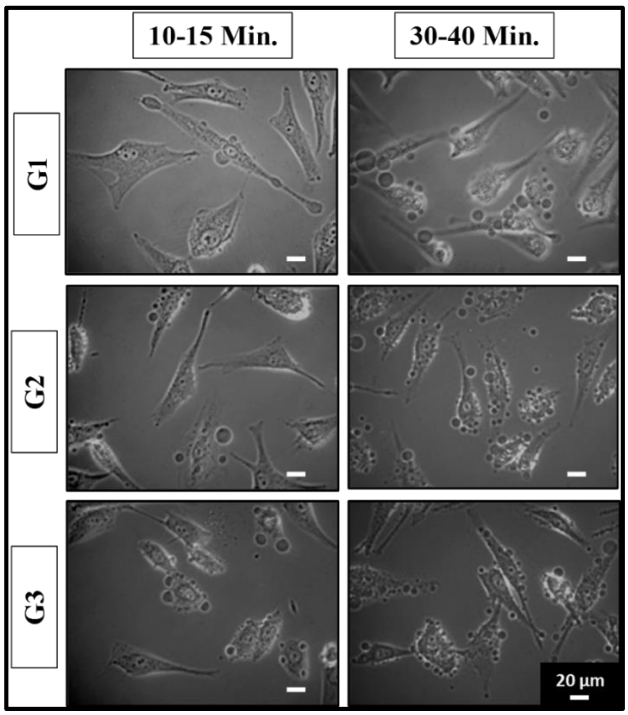

Figure 5.2: Phase Contrast Microscopy of the generating GPMVs

Table 5.1: Size analysis by Image J software

Sample	Average diameter (µm)	Range (µm)
G1	**10.9**	**20-6**
G2	**8.4**	**15-4**
G3	**9.8**	**16-7**

The cells were treated with the blockers for the optimized time period which was 30 min. After the blocker treatment the GPMV working solution was added and the flasks along with the GPMV buffer was incubated for 45 min. For GPMV to come in suspension it was kept on an orbital shaker. Time dependent microscopy was done to analyse the size and effect of blocker on GPMV synthesis. It was clearly observed that the blockers resist the blebbing of the membrane. The average size of the GPMV after the cells were treated with the cytochalasin-D and methyl-β-cyclodextrin was significantly smaller as compared to the GPMV generated from the untreated MG-63 cells. The size of the GPMV was calculate using the NIH Image J software.

5.2.2 Environmental Scanning Electron Microscopy(ESEM)

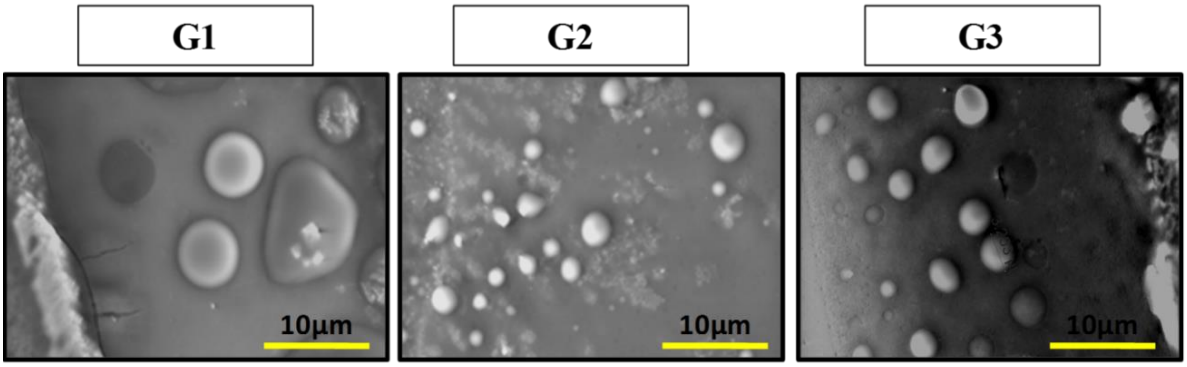

Figure 5.3: Analysis of generated GPMVs under Environmental scanning electron microscope

Table 5.2: Size analysis of the GPMVs using NIH Image J software

Sample	Average diameter (µm)	Range (µm)
G1	**7.8**	**12-4**
G2	**3.2**	**6-2**
G3	**3.9**	**5-2**

The size of the GPMV calculated by analysing it using the Image J software by NIH. The size of GPMV was observed to be reduced this is because of the overnight drying of the sample before observing the sample by scanning electron microscopy.

5.2.3 Fluorescence Microscopy

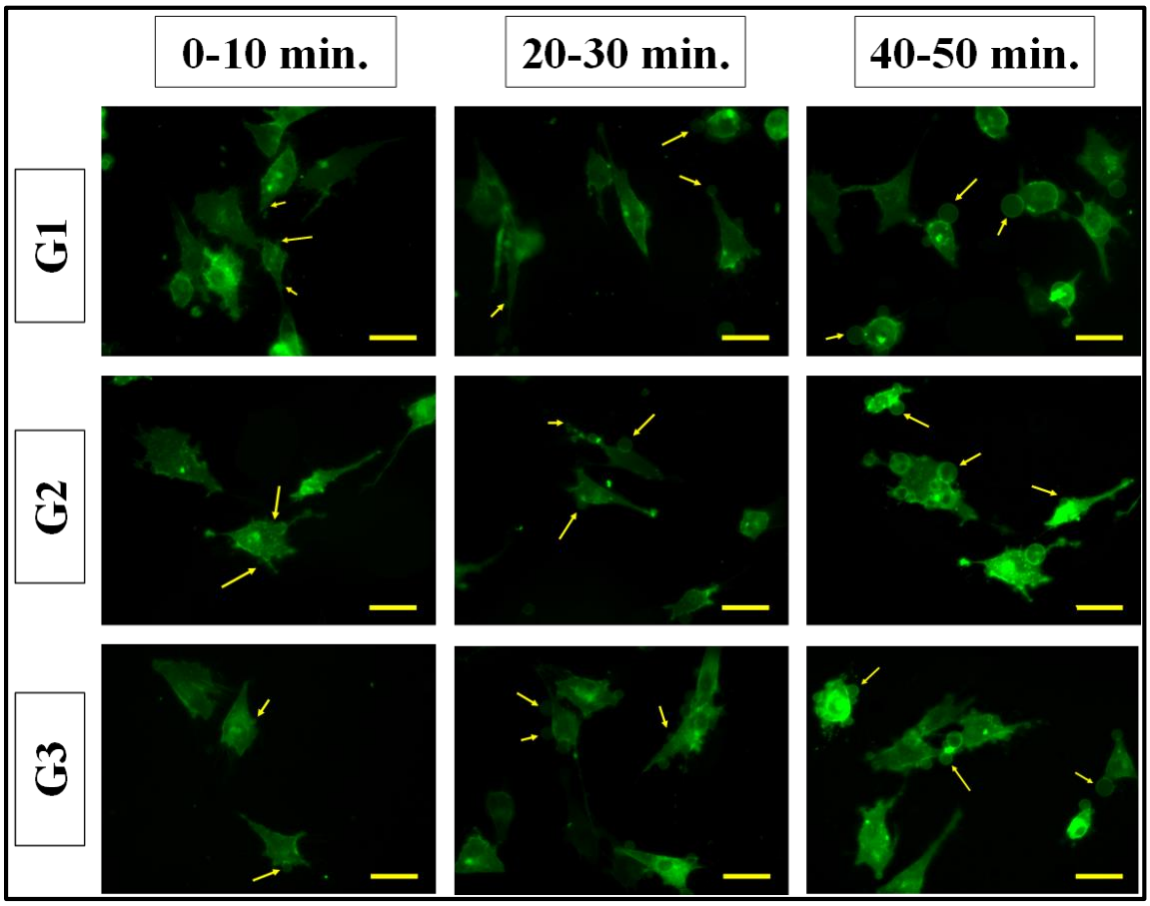

Figure 5.4: Time dependent fluorescence microscopy of the GPMVs stained with DiO

The size and kinematics of the synthesis of GPMV was significantly affected by the treating the cells with the blockers prior to the generation of the GPMV. As cytochalasin-D affects the cytoskeleton made by myosin-actin filaments the induction of membrane blebbing was inhibited by the treatment thus, affecting the size of the vesicles. The cholesterol sequester treatment i.e. treatment with methyl-β-cyclodextrin the membrane fluidity is decreased thereby reducing the membrane fluidity and inhibiting the synthesis of GPMV. Different kind of membrane Blebbing kinetics was observed by performing the time-dependent microscopy of the GPMV stained with DiO and observed under fluorescence microscope.

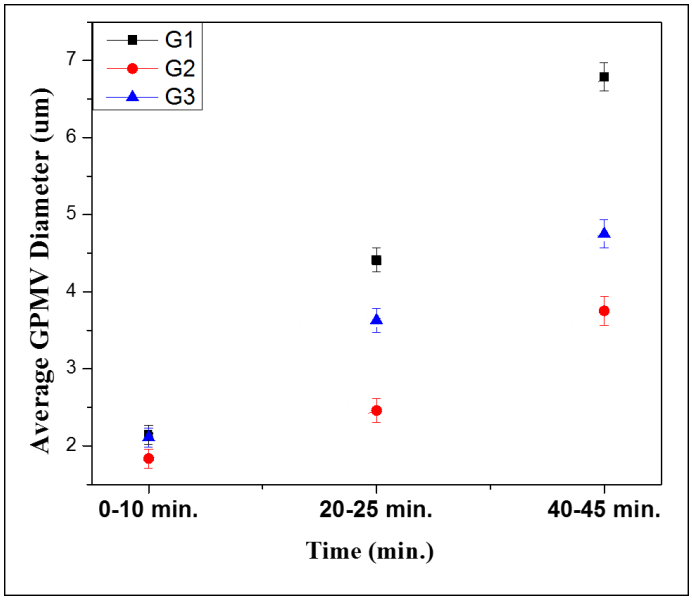

Figure 5.5: Kinetics of GPMV generation

Size analysis was done using the NIH Image J software and the graph was plotted in the origin to analyse the kinetics of GPMV generation. It was observed that the cells treated with the cytochalsin-D resist the membrane blebbing and GPMV synthesized are of smallest size.

5.3 Analysis of membrane flipping of the GPMVs by Annexin V

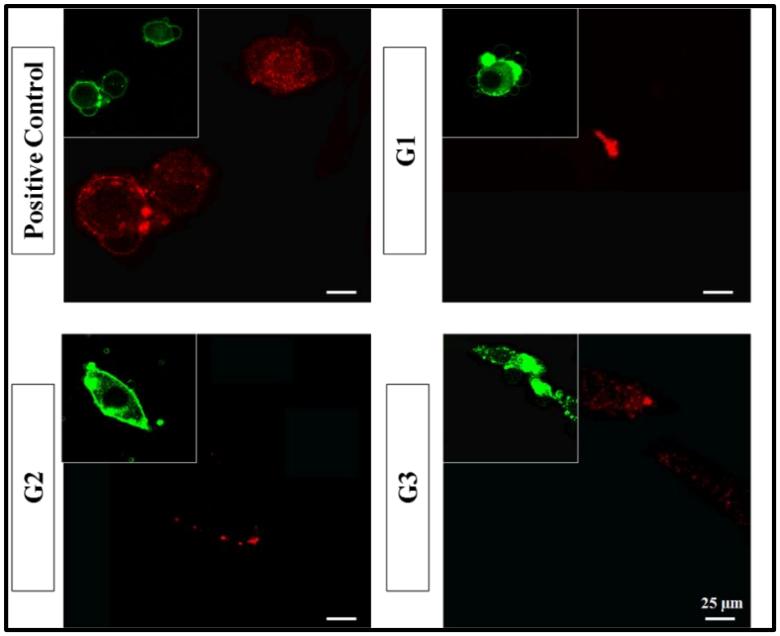

Figure 5.6: Analysis of membrane flipping by staining the GPMVs with DiO and Annexin-V by confocal microscopy

The pro-apoptotic cells exhibit a unique property of membrane flipping the phospholipid phosphatidylserine from the cytosolic side of the cell membrane flips to the outer side of the membrane. The annexin-V binds with the phosphatidylserine and phycoerythrin fluorescent dye is attached with the annexin-V and gives red colour. Here, we have induced apoptosis in the MG-63 cells by using the hydrogen peroxide as apoptosis inducer.

The GPMV was produce for all the three samples. No significant membrane flipping was observed in blebs formed in the presence of GPMV working solution. Whereas, in the case of positive control membrane flipping was observed in the apoptotic blebs.

5.4 Analysis of membrane integrity by Propidium Iodide

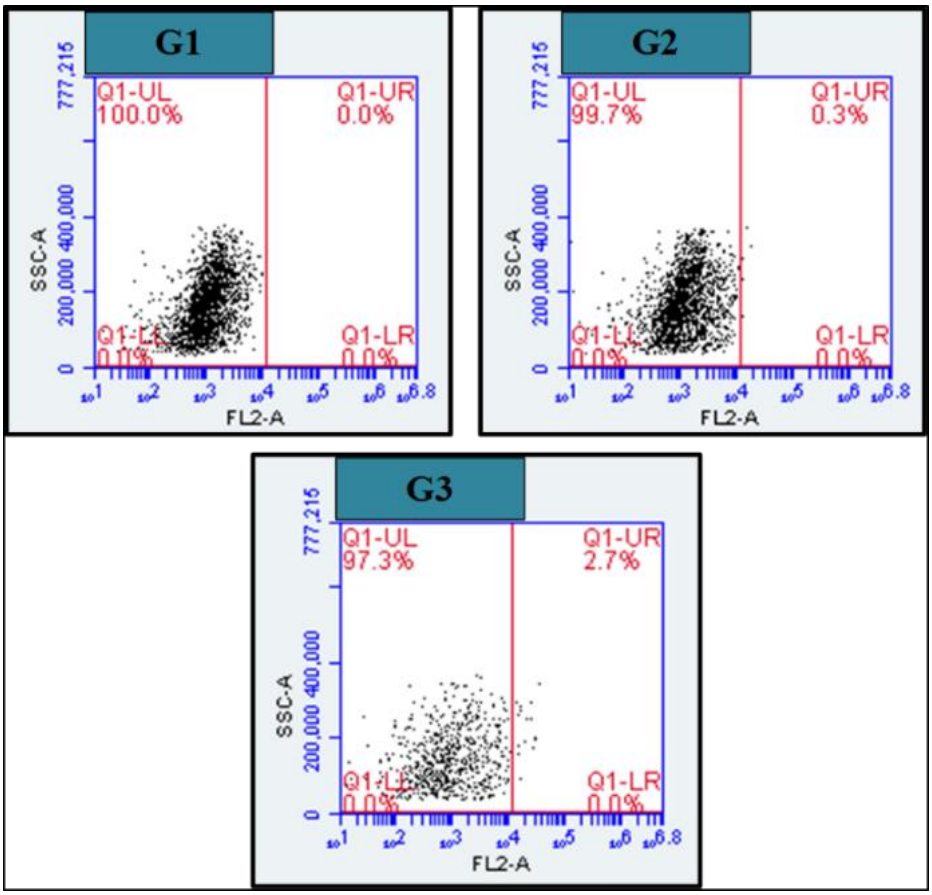

Figure 5.7: Analysis of the integrity of the membrane by flow cytometry of the generated GPMVs by using Propidium Iodide

The membrane integrity of the GPMV was analysed using the propidium iodide dye as it will only penetrate the cell when the membrane is disintegrated and the dye will bind with the DNA. Here in all the three cases as observed in the figure 5.7 was less than 3% of population has been observed to take up the stain which is not significant. That proves that

the membrane is not leaky it's a complete intact vesicle and the integrity of the membrane was maintained in the synthesis of GPMV.

5.5 Analysis of membrane Complexity by Flow Cytometry

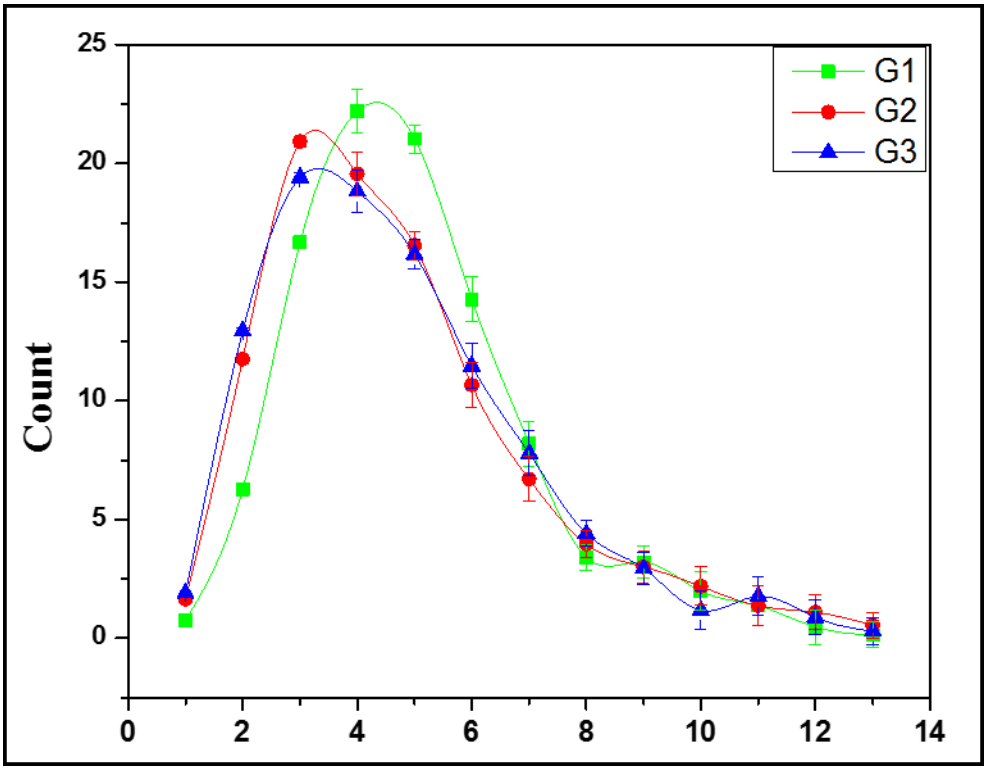

Figure 5.8: Analysis of the membrane complexity by flow cytometry of the generated GPMVs by flow cytometry

Table 5.3: Size analysis of the GPMVs using Image J software

Sample	No. of GPMV/µl
Control	10
Cyto-D	1
MβCD	2

The complexity of the membrane was analysed using the forward scatter plot of flow cytometry as shown in figure 5.8. The GPMVs generated after the blocker treatment shows a distinct and clear difference in the composition of the plasma membrane vesicles. The number of GPMVs generated was very less in the cells treated with the blockers. As the blocker treatment inhibits the blebbing of the membrane. The resistance offered by the blocker treatment was clearly observed by the flow cytometry analysis.

5.6 Characterization of the Lipids Extracted from the Giant Plasma Membrane Vesicles

5.6.1 Biochemical Assay

5.6.1.1 Cholesterol Estimation Assay

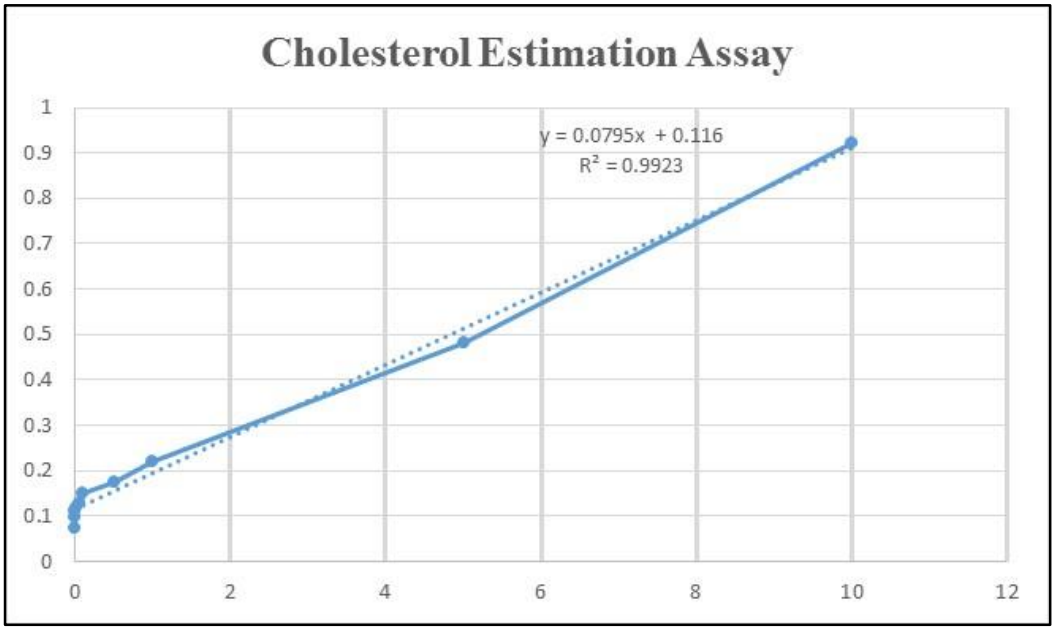

Figure 5.9: Standard curve for the analysis of the cholesterol present in the lipid extracted from the GPMV by UV-Visible spectroscopy

Table 5.4: Concentration of Cholesterol estimated by using the equation derived from standard curve

Samples	Concentration (mg/ml)
G1	**1.107**
G2	**1.648**
G3	**0.641**

As the literature suggests that the methyl-β-cyclodextrin is cholesterol sequester, it removes the cholesterol present in the membrane by forming a dimer complex with the cholesterol thus, affecting its fluidity. The biochemical assay was used to estimate the amount of cholesterol present in the lipid extracted by using the chloroform: methanol extraction

process. Table 5.4 shows the concentration of the cholesterol present using the equation derive from the standard curve. It was observed that the lipid extracted from the GPMV generated after the cells were treated with methyl-β-cyclodextrin was significantly lower than the other two samples. This proves that the methyl-β-cyclodextrin works as the cholesterol sequester.

5.6.1.2 Phosphorus estimation Assay

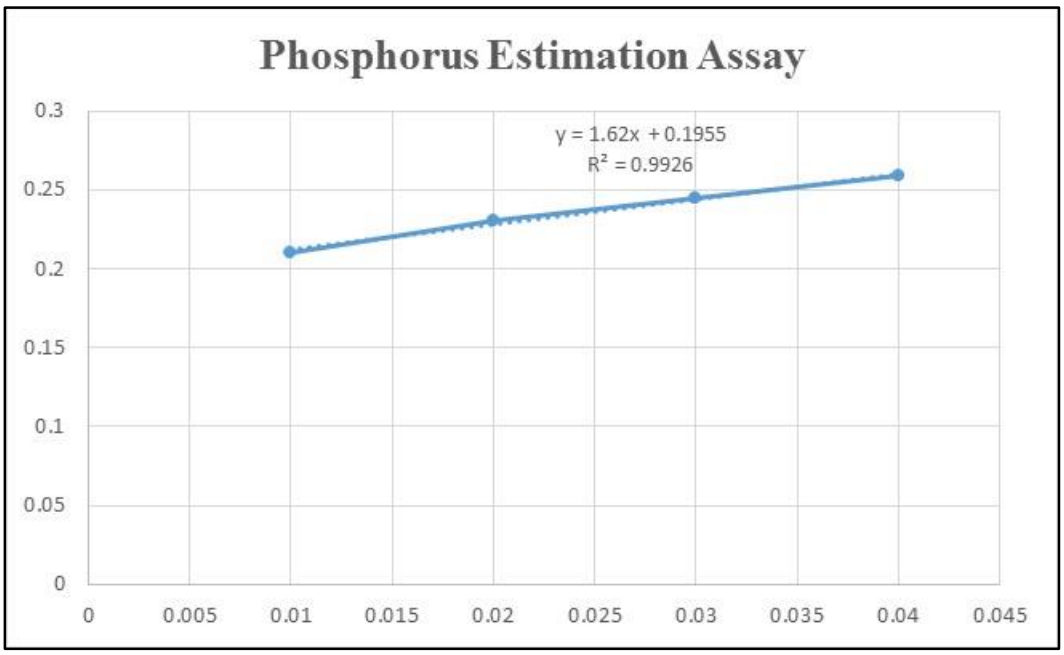

Figure 5.10: Standard curve for the analysis of the phosphorus present in the lipid extracted from the GPMV by UV-Visible spectroscopy

Table 5.5: Concentration of phosphorus lipid was calculated using the equation derived from standard curve

Samples	Concentration(µmolar)
G1	**0.0157**
G2	**0.0182**
G3	**0.0361**

The phosphorus in the total phospholipid was calculated using the equation derived after plotting the standard curve for the phosphorus estimation. The lipid extraction was done

using the chloroform methanol lipid extraction method. The phosphorus concentration was significantly higher in sample G3 of the lipid extracted from the GPMV synthesized after the cells were treated methyl-β-cyclodextrin as compared to sample G1 and G2 as shown in table 5.5.

5.6.2 Nuclear Magnetic Resonance spectroscopy

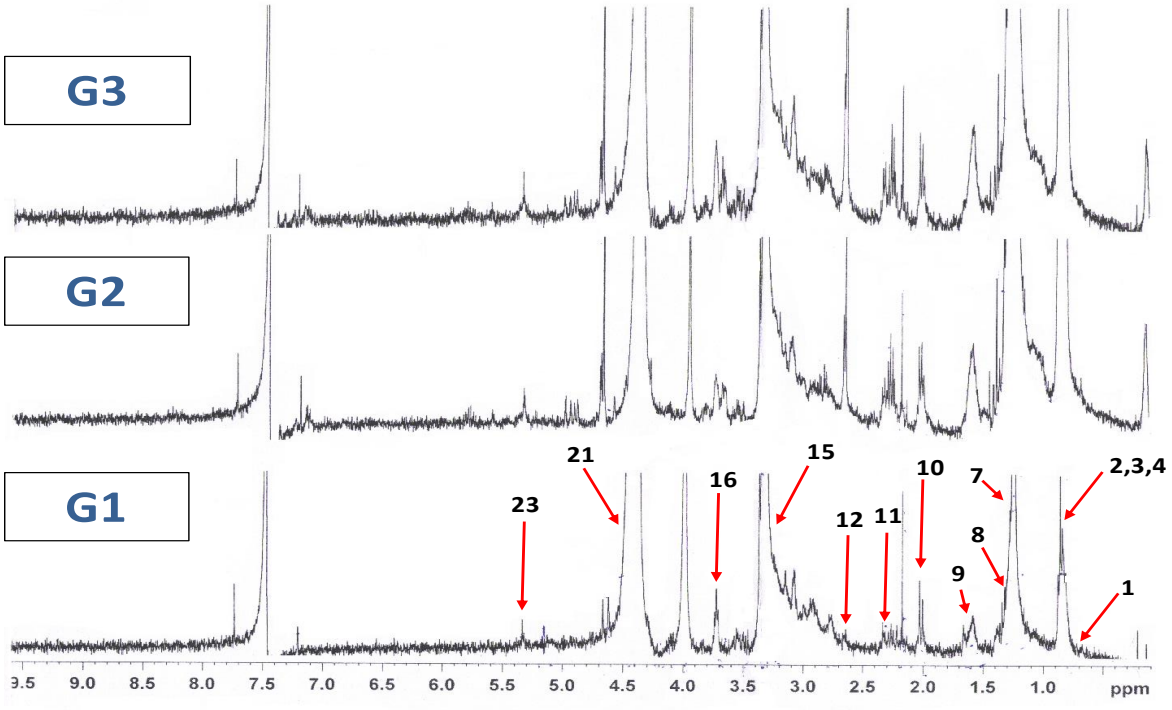

Figure 5.11: NMR spectroscopy of the lipid samples extracted from the generated GPMV

The nuclear magnetic resonance spectroscopy was performed to analyse the phospholipids quantitatively and qualitatively present in the lipid extracted from the GPMVs using the chloroform methanol extraction method. The peaks were assigned in the figure 5.11 using the reference shown in figure 5.12 as shown below which was described by [24]. We were able to assign nearly all the peaks this proves that the lipid composition of GPMV is same lipid composition of the cell membrane. The areas under the peak was used to analyse the data quantitatively. The peaks clearly show a difference in the phospholipid composition in the samples G1, G2 and G3. This validates that the blocker treatment affects the complexity as well as composition of the GPMV. The analysis of the peaks was done using the NMR spectroscopy software.

Resonance number	Chemical shift (ppm)	Assignments
1	0.70	$-C_{18}\mathbf{H}_3$ in total cholesterol
2	0.86/0.88	$-C_{26}\mathbf{H}_3/-C_{27}\mathbf{H}_3$ in total cholesterol
3	0.88	$-\mathbf{CH}_3$ in fatty acyl chain
4	0.925	$-C_{21}\mathbf{H}_3$ in free cholesterol
5	1.012	$-C_{19}\mathbf{H}_3$ in free cholesterol
6	1.04	$-C_{19}\mathbf{H}_3$ in esterified cholesterol
7	1.27	$-(\mathbf{CH}_2)_n$ in fatty acyl chain
8	1.32	$=CHCH_2\mathbf{CH}_2(CH_2)-$ in fatty acyl chain
9	1.61	$-CO-CH_2\mathbf{CH}_2-$ in fatty acyl chain
10	2.05	$-\mathbf{CH}_2HC=$ in fatty acyl chain
11	2.34	$-CO-\mathbf{CH}_2-$ in fatty acyl chain
12	2.82	$=CHCH_2CH=$ in fatty acyl chain
13	3.21	$-N^+(\mathbf{CH}_3)_3$ in SM head group
14	3.22	$-N^+(\mathbf{CH}_3)_3$ in PC head group
15	3.37	Solvent (methanol)
16	3.61	$-\mathbf{CH}_2N^+(CH_3)_3$ in PC or SM head group
17	4.01	$>C_3\mathbf{H}_2$ in glycerol backbone of PL
18	4.17	$>C_1\mathbf{H}_2$ in glycerol backbone of PL and TG
19	4.25	$-CH_2\mathbf{CH}_2N^+(CH_3)_3$ in PC or SM head group
20	4.34	$>C_1\mathbf{H}_2$ and $>C_3\mathbf{H}_2$ in glycerol backbone of TG
21	4.44	$>C_1\mathbf{H}_2$ in glycerol backbone of PL
22	5.20-5.28	$>C_2\mathbf{H}$ in glycerol backbone of PL and TG
23	5.35	$-\mathbf{HC}=\mathbf{CH}-$ in fatty acyl chain

Figure 5.12: Reference for NMR spectroscopy of the lipid samples extracted from the generated GPMV [24]

5.7 Analysis of the Fusion efficiency of hMSCs with GPMV

5.7.1 Confocal microscopy

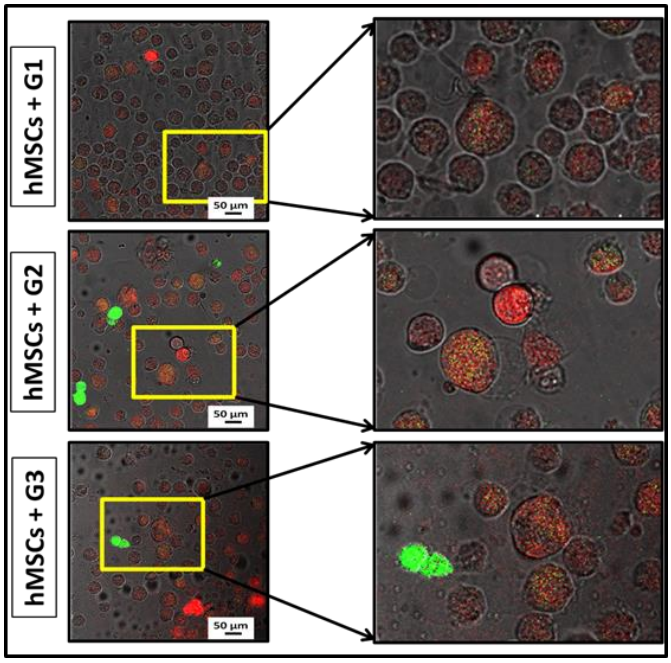

Figure 5.13: Fusion analysis of the hMSCs stained with cell tracker red and GPMV Stained with the DiO vybrant dye

As previously described in the schematic that the GPMVs were stained with the DiO and hMSCs were stained with the cell tracker red. If the fusion is induced, then the fused hMSCs will give a orangish-yellowish shade of colour. As after the fusion the GPMVs stain that is vybrant Dio will leach out and spread in the cell membrane. Figure 5.13 clearly shows that the GPMVs in all the three samples G1, G2 and G3 have undergone the fusion with the hMSCs. This proves our hypothesis that the GPMVs can fuse with the live cells. Further as shown in the Figure 5.14 the flow cytometric analysis was done to evaluate the fusion efficiency. It was observed that more than 15% of the cells were fused with the GPMVs as they take up the GPMVs stained with the DiO and shows fluorescence. From figure 5.14 and Figure 5.15 it is established that the GPMVs fusion with the hMSCs can be achieved by centrifugation based method.

5.7.2 Flow Cytometry

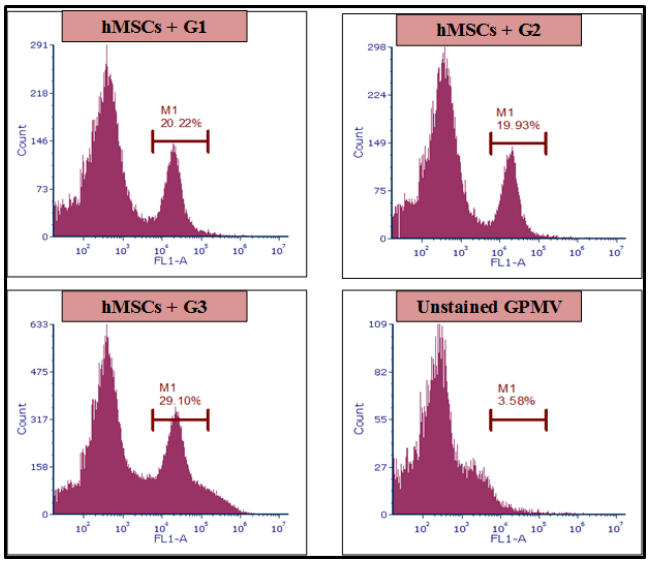

Figure 5.14: Fusion analysis of the hMSCs stained with cell tracker red and GPMV Stained with the DiO vybrant dye by flow cytometry

5.8 Cell proliferation study of the hMSCs Cultured in presence of GPMV

To evaluate that whether the presence of GPMVs or GPMV buffer in very less quantity is having any toxic effects on the cell growth and proliferation the Following studies was performed on the hMSCs after the cells were fused with the GPMV and then cultured in the presence of GPMVs. The phase contrast microscopy images in figure 5.15 shows that

the hMSCs have attain their natural morphological shape after the incubation for 24 h and 96 h. Proving that after the fusion the cells will proliferate naturally. Fusion did not affect the morphology of the cells.

5.8.1 Phase Contrast Microscopy

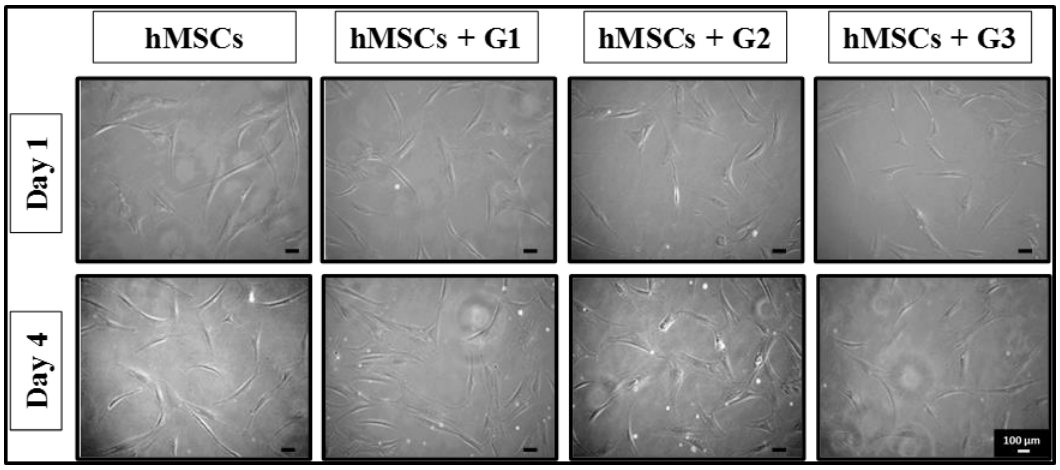

Figure 5.15: Phase contrast microscopy of the hMSCs fused with the GPMV

5.8.2 Confocal Microscopy

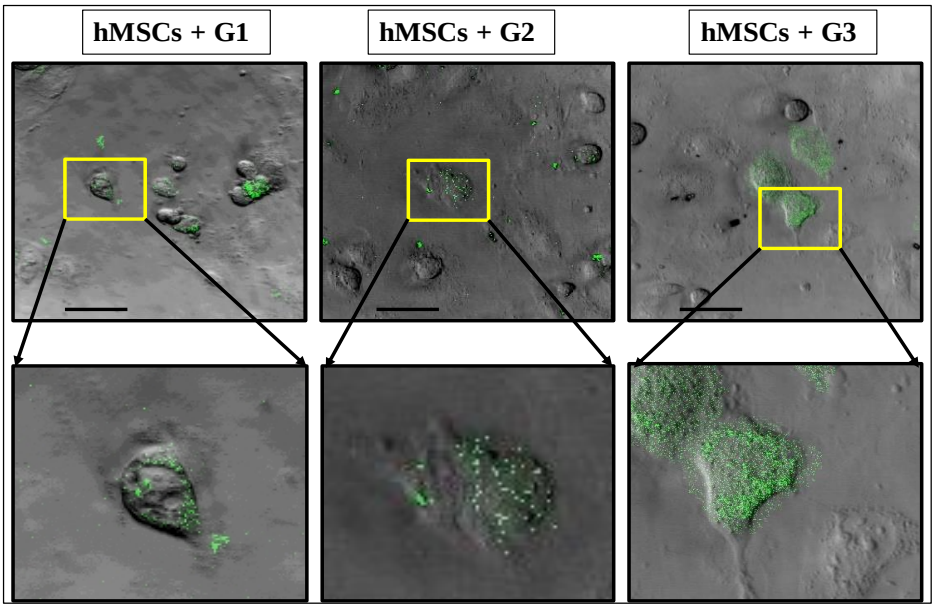

Figure 5.16: Confocal microscopy of the hMSCs fused with the GPMV stained with DiO

Fusion was performed by a centrifugation based method, the GPMVs were stained with vybrant DiO. After the fusion, the cells with GPMVs were seeded in the prepared confocal wells. After 12 h of incubation at 37ºC at 5% CO_2 the cells were observed under the lieca SP8 confocal microscope and images were analysed using lieca LasX software.

5.8.3 MTT assay

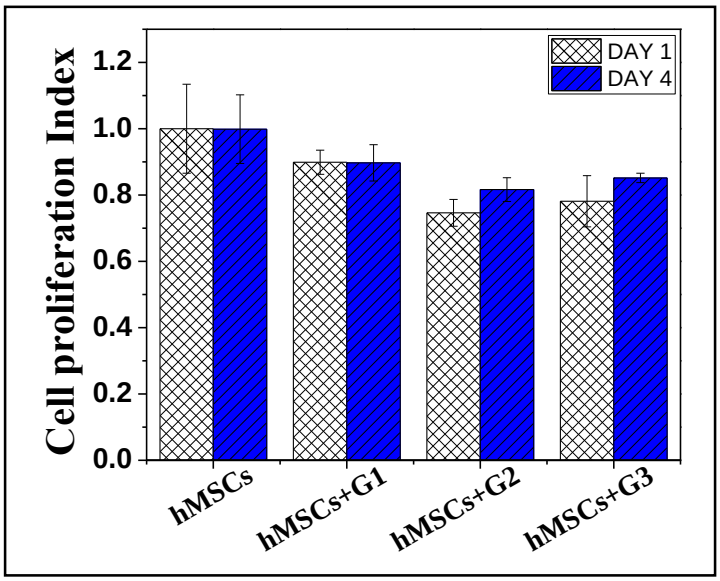

Figure 5.17: Cell proliferation study using MTT assay of the hMSCs fused with the GPMV stained with DiO

The cell proliferation index calculated from the MTT assay shows the cell viability of more than 75% in all the three samples G1, G2 and G3. The cells after the fusion is performed are proliferating without any cytotoxic effect of GPMVs or GPMV buffer on the cells as shown in figure 5.17.

5.8.4 Propidium iodide based live/dead assay

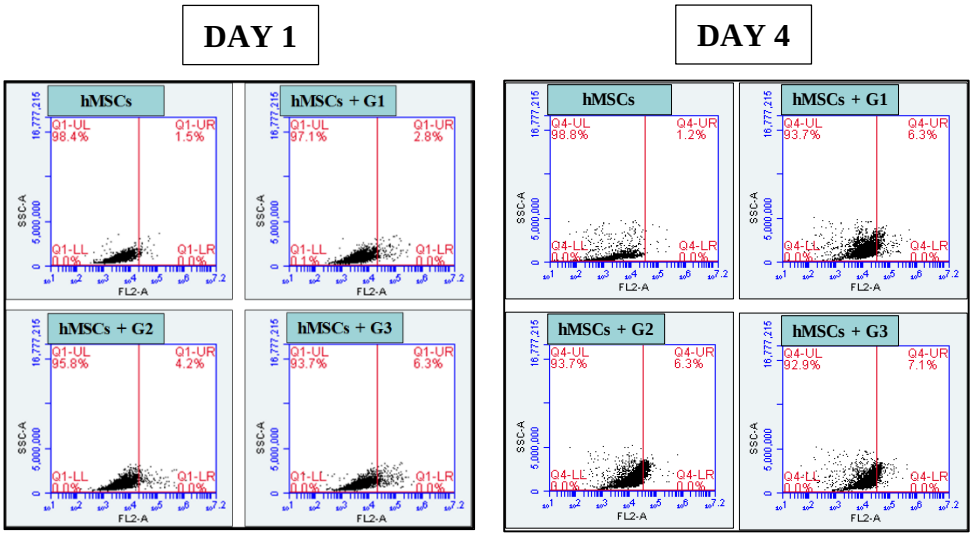

Figure 5.18: Propidium Iodide based livedead assay, analysed using flow cytometry

Flow cytometric analysis was done after staining the cells with the propidium iodide (PI) stain. PI will bind with DNA only after it enters into the cells after the membrane disintegration. This differentiate between the live and dead cell population present in the sample. The live/dead assay was performed for day 1 and day 4. No distinct dead cell population of hMSCs was observed after the fusion of hMSCs with the G1, G2 and G3.

5.9 Osteogenic differentiation study of the hMSCs cultured in presence of GPMV

5.9.1 Phase contrast microscopy

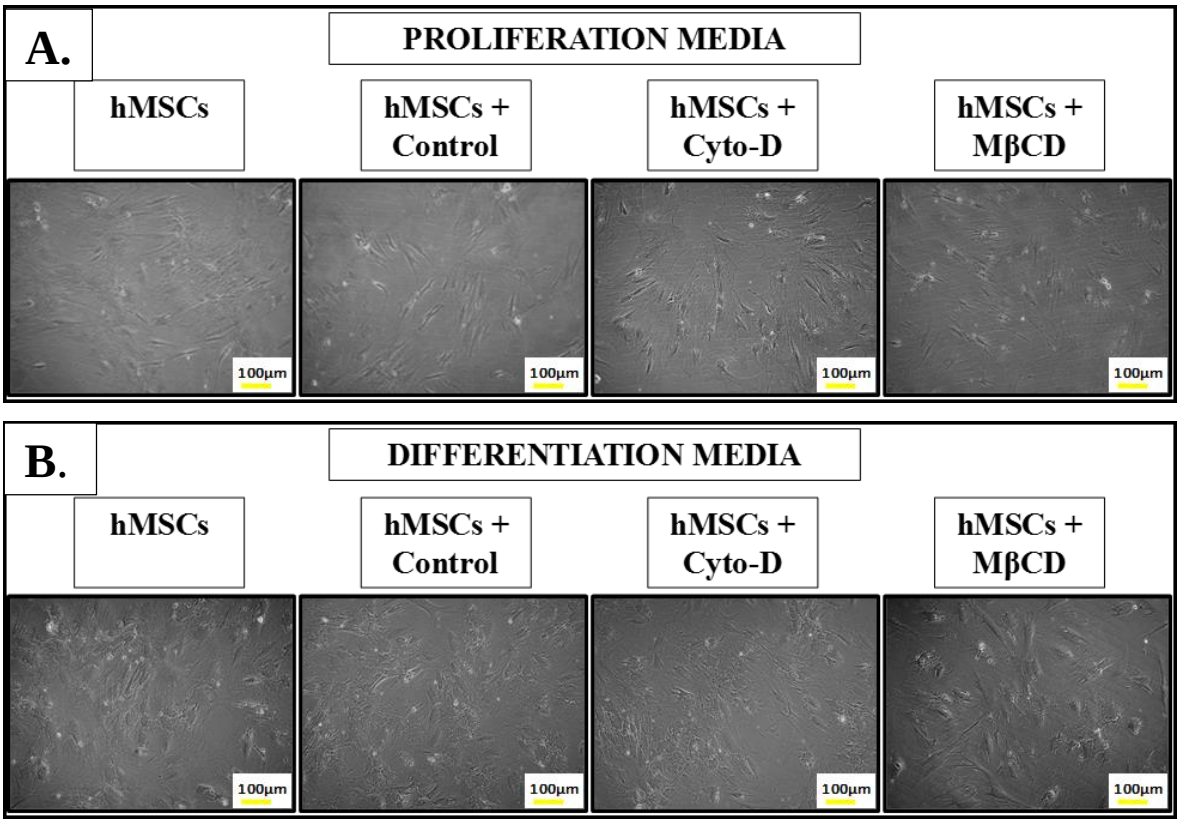

Figure 5.19: Phase contrast microscopy of the hMSCs cultured in the presence of the GPMV and the growth medium used was A) Expansion medium and B) Differentiation media

Phase contrast microscopy was performed to observe the morphological changes in the MSCs when cultured in expansion median and differentiation media. Significant difference in the morphology of the cells was observed as we can see in the figure 5.19. this proves that the differentiation media induce some morphological changes when the it induces the osteogenic differentiation of the hMSCs.

5.9.2 Alkaline phosphatase assay

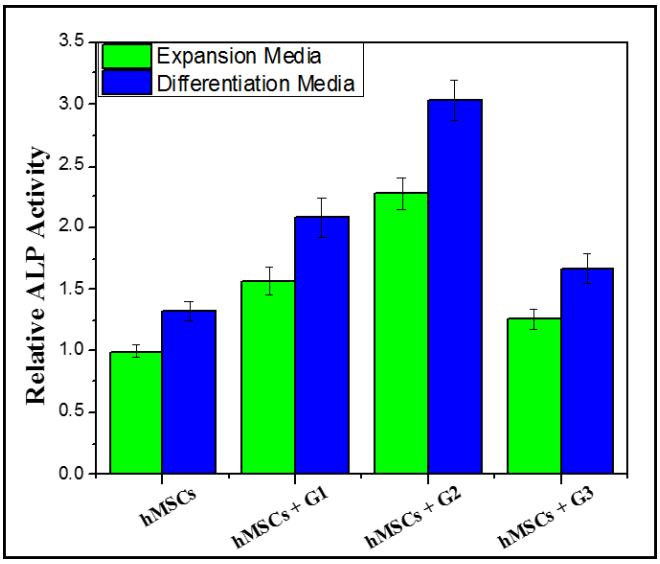

Figure 5.20: Analysis of alkaline phosphatase activity of the hMSCs cultured in the presence of the GPMV in expansion and differentiation media

The alkaline phosphatase is the enzyme that is directly proportional to the bone metabolism that means the cells produce this when the osteoclast differentiates into osteocyte. The ALP quantitative analysis shows that the when the hMSCs fused with GPMV s and cultured in the presence of GPMVs they differentiate into osteogenic lineage. hMSCs with G2 shows the highest amount of ALP activity as compared to hMSCs + G1 and hMSCs + G3, but all three of them was significantly higher than the level of expression in the hMSCs.

5.9.3 Alizarin red staining

The Alizarin red staining (ARS) was performed to analyse the matrix deposition or mineralization of the cells. As compared to hMSCs all the three samples hMSCs + G1, hMSCs + G2 and hMSCs + G3 shows higher level of matrix deposition on the cells. In differentiation media this value was relatively higher as observed by the phase contrast microscopy and then confirmed by dissolving the matrix deposited by using cetyl pyrimidine chloride solution and taking the absorbance at 550 nm.

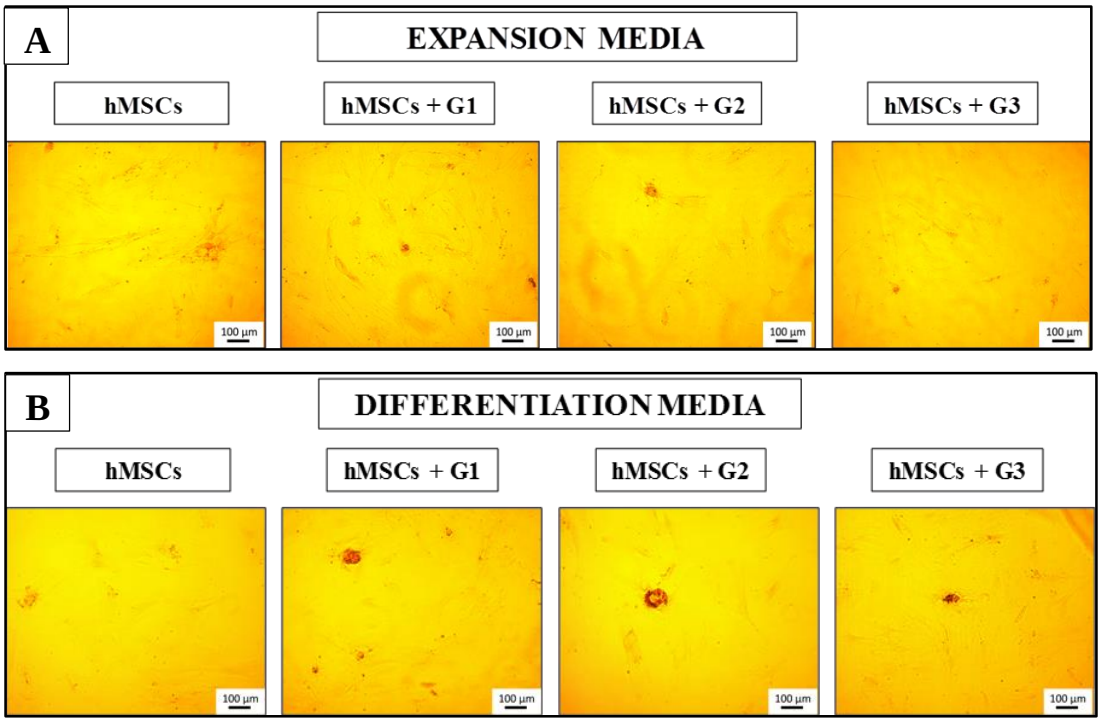

Figure 5.21: Phase Contrast microscopy of matrix deposition on the hMSCs cultured in the presence of the GPMV in A) Expansion media and B) Differentiation media

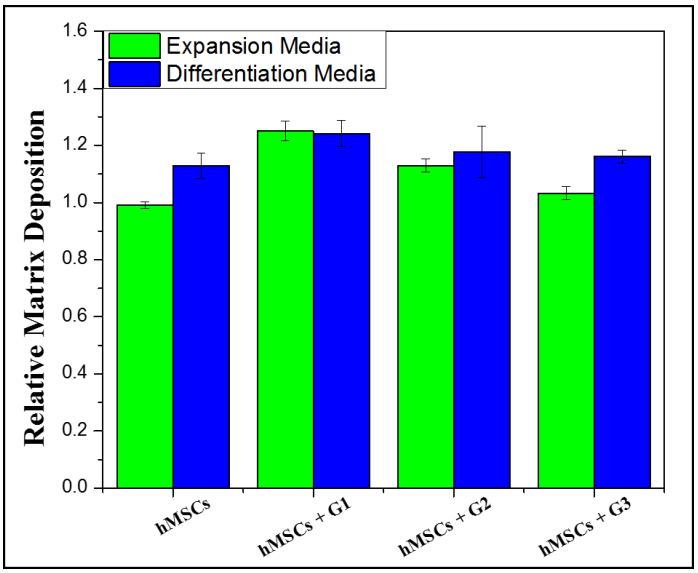

Figure 5.22: Analysis of matrix deposition on the hMSCs cultured in the presence of the GPMV in expansion and differentiation media

By quantitative assay also it was proved that the mineralization of the bone matrix was higher in the samples, though no significant difference was observed in between the samples.

5.9.4 Collagen estimation Assay

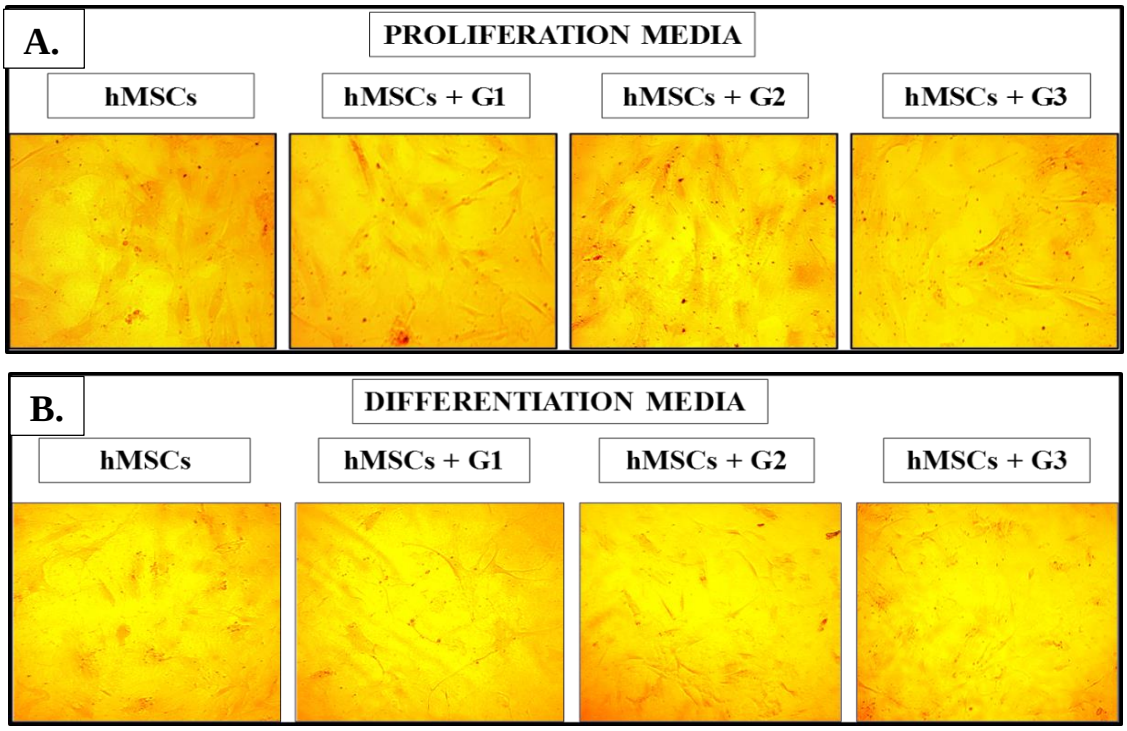

Figure 5.23: Phase contrast microscopy of the collagen synthesis by the hMSCs cultured in the presence of the GPMV in A) Expansion media and B) Differentiation media

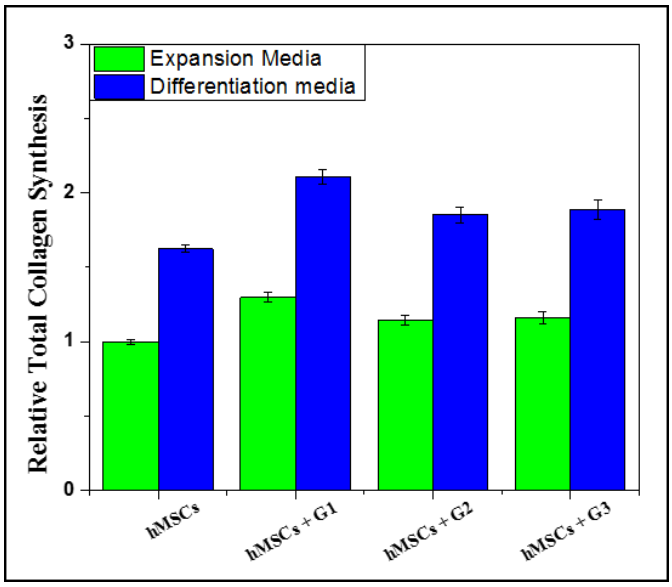

Figure 5.24: Analysis of the total collagen synthesis by the hMSCs cultured in the presence of the GPMV in expansion and differentiation media

Collagen is required to provide bone strength. The synthesis of collagen helps in bone remodelling and repair. The study of collagen synthesis was done by using the phase contast

microscopy then dissolving the sirius dye stain in the sodium hydroxide solution for quantitative analysis and the absorbance was measured at 550 nm. figure 5.23 and figure 5.24 both indicates the higher synthesis of total collagen by the hMSCs fused with the GPMVs and then cultured in the presence of GPMVs as compared to hMSCs. Collagen is an early osteogenic marker its increase expression proves that the GPMVs induces osteogenic-differentiation of the hMSCs.

5.9.5 Reverse Transcriptase – Polymerase Chain Reaction

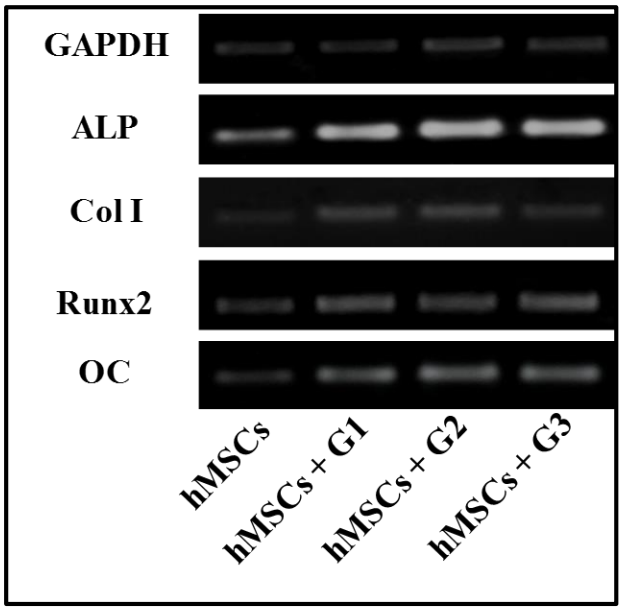

Figure 5.25: Bands of the cDNA observed after performing the RT-PCR analysis of the mRNA isolated from the cells cultured in the expansion media

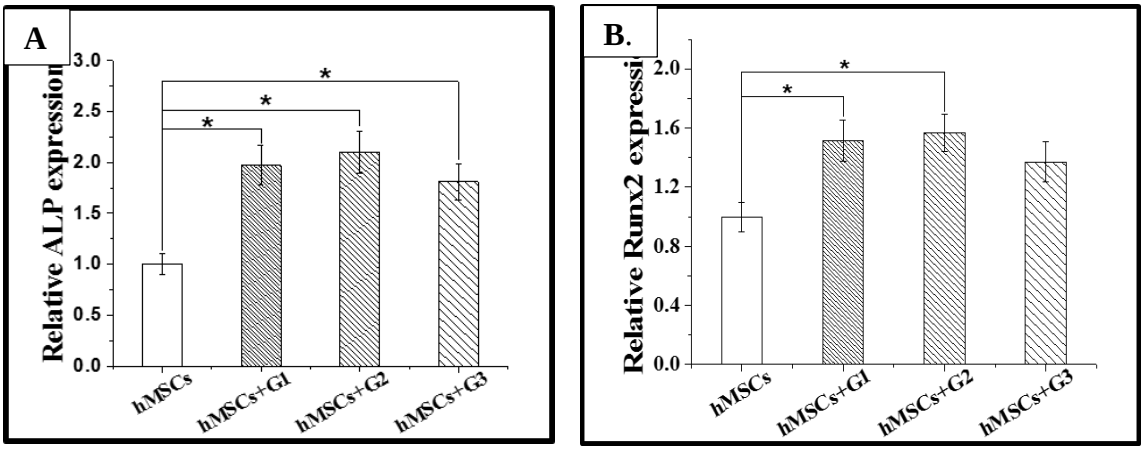

Figure 5.26.1: Intensity analysis of the cDNA observed after performing the RT-PCR analysis using NIH Image J software. A) Relative ALP expression B) Relative Runx2 expression

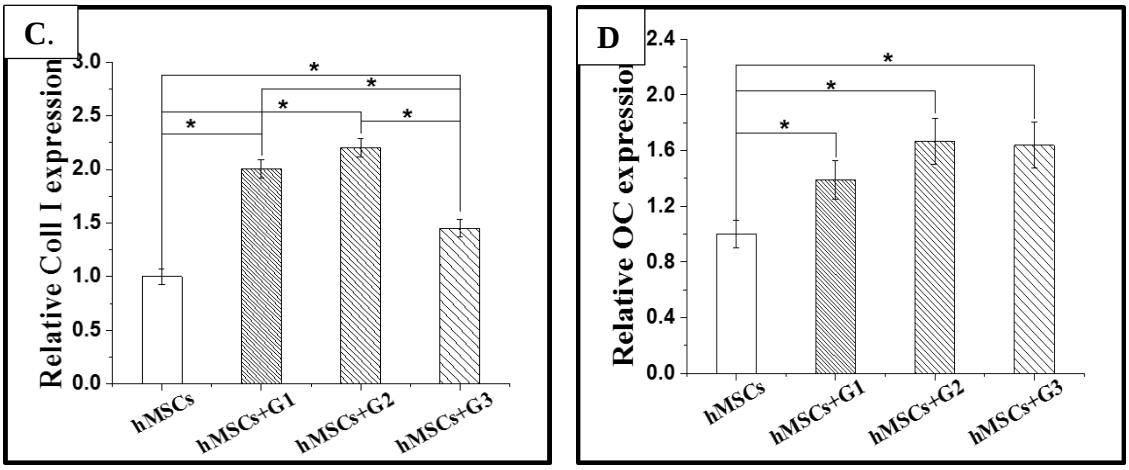

Figure 5.26.2: Intensity analysis of the cDNA observed after performing the RT-PCR analysis using NIH Image J software C) Relative Col 1 expression D) Relative OC expression

For the RT-PCR analysis we selected four different osteogenic markers: alkaline phosphatase, collagen 1, Runt related transcription factor and octeocalcin. All the early and late osteogenic markers show that the hMSCs fused and cultured in the presence of GPMVs have a significantly higher amount of markers expression as compared to MSCs. As shown it the figure 5.26.1 and 5.26.2 Thus, it's been concluded that GPMVs directs hMSCs to osteogenic lineage.

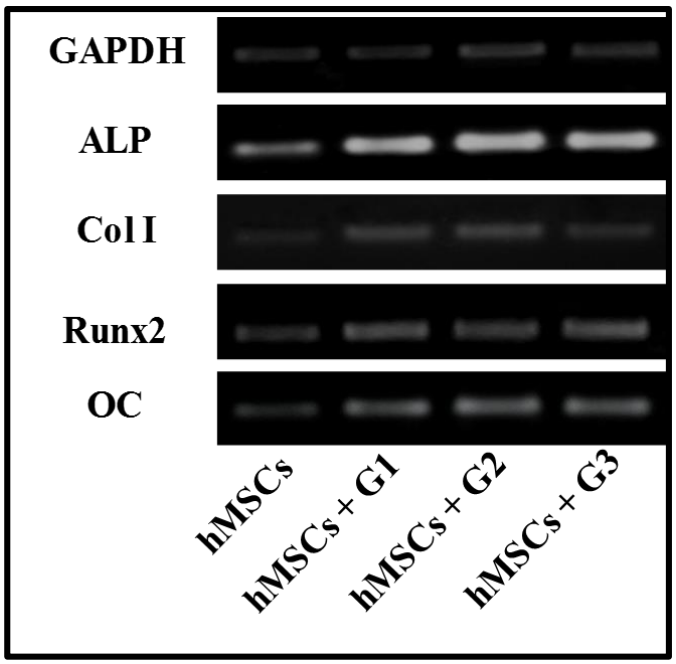

Figure 5.27: Bands of the cDNA observed after performing the RT-PCR of the isolated mRNA cultured in the presence of differentiation media

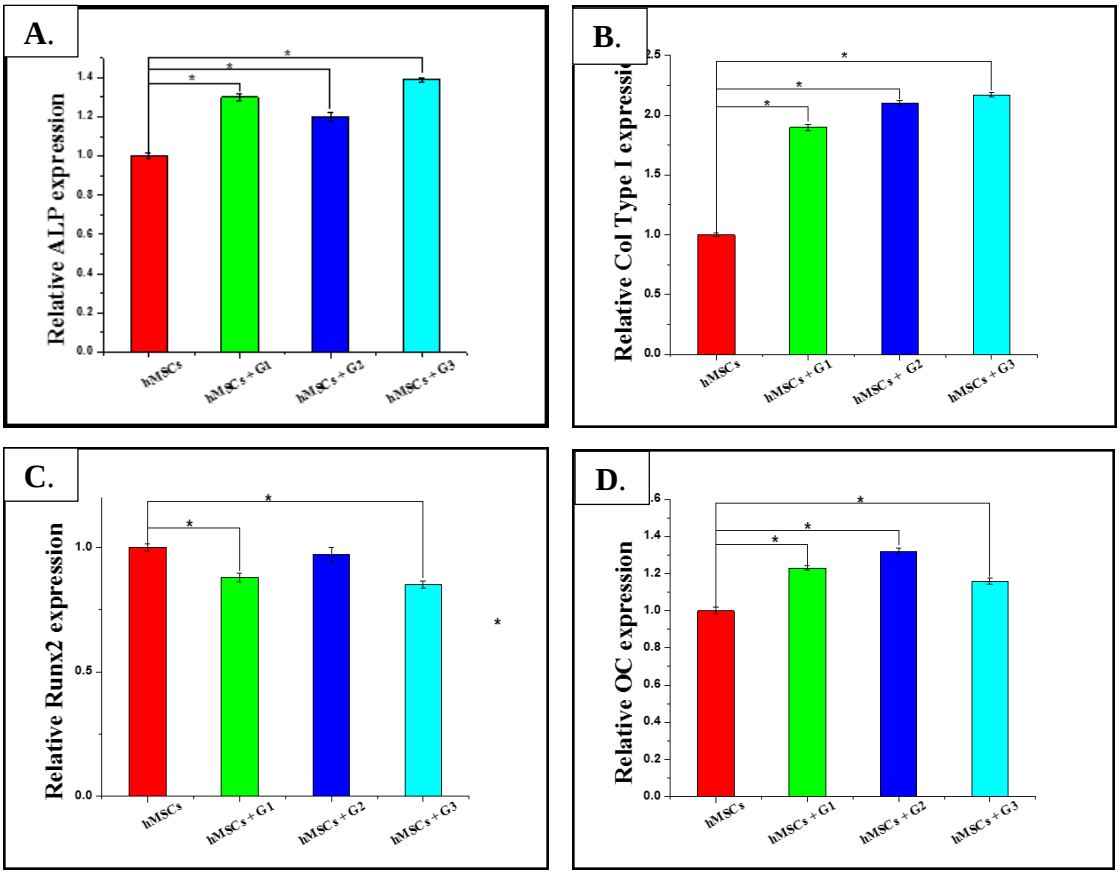

Figure 5.28: Intensity analysis of the cDNA observed after performing the RT-PCR of the hMSCs cultured in the differention media and the analysis was done using Image J software a) Relative ALP expression B) Relative Col 1 expression C) Relative Runx2 expession D) Relative OC expression

For the RT-PCR analysis we selected four different osteogenic markers: alkaline phosphatase, collagen 1, runt related transcription factor and octeocalcin. After 14 days the mRNA was isolated and one step RT-PCR was performed. The results were analysis by running the 8% agarose gel with 1% ethidium bromide. All the early and late osteogenic markers show that the hMSCs fused and cultured in the presence of GPMVs have a significantly higher amount of markers expression as compared to hMSCs. As shown it the Figure 5.28. Thus, it's been concluded that GPMVs directs hMSCs to osteogenic lineage even in the presence of differentiation media used as growth medium.

5.10 Osteogenic differentiation study of the hMSCs cultured in presence of GPMV buffer

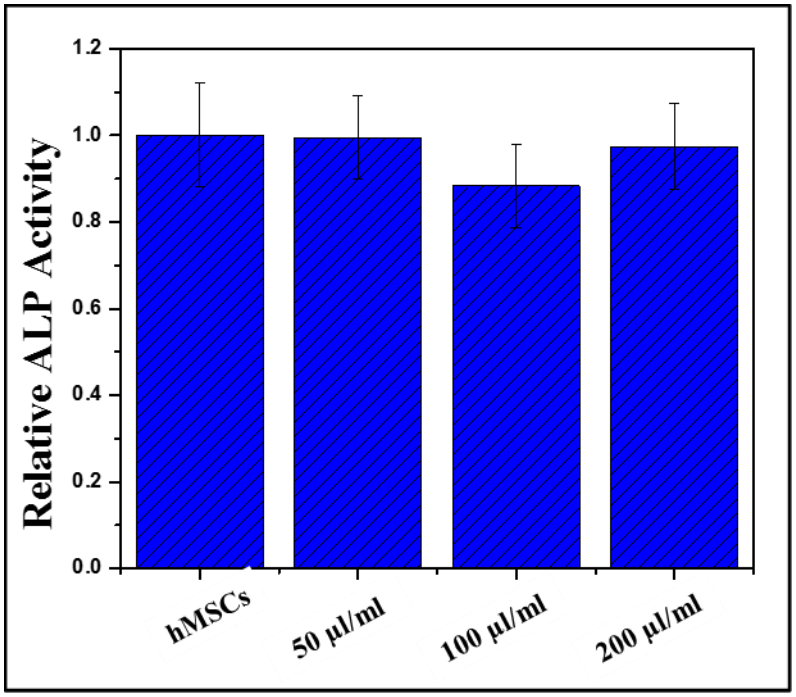

Figure 5.29: ALP activity analysis of the hMSCs cultured with three different concentration of GPMV buffer

When we seed the cells for the downstream analysis, a very little of GPMV buffer also enters into the system. So, to remove that possibility that the GPMV working soluton may induce the differentiation of the hMSCs to osteoblasts. Three different concentration of the GPMV buffer was used. The study concludes that the GPMV buffer does not induce the diffrentiation of hMSCs.

5.11 VEGF expression in the hMSCs cultured in presence of GPMV

For the bone to remodel or repair both osteogenesis and angiogenesis should happen simultaneously. The release of VEGF in the medium to induce the growth of proper vascular system in the tissue or graft. Here, we observe that the VEGF expression in the hMSCs fused and then cultured in the presence of GPMV was lower than the MSCs as shown in figure 5.30 this may be because the GPMV generated does not carried the signalling molecule required for the VEGF expression.

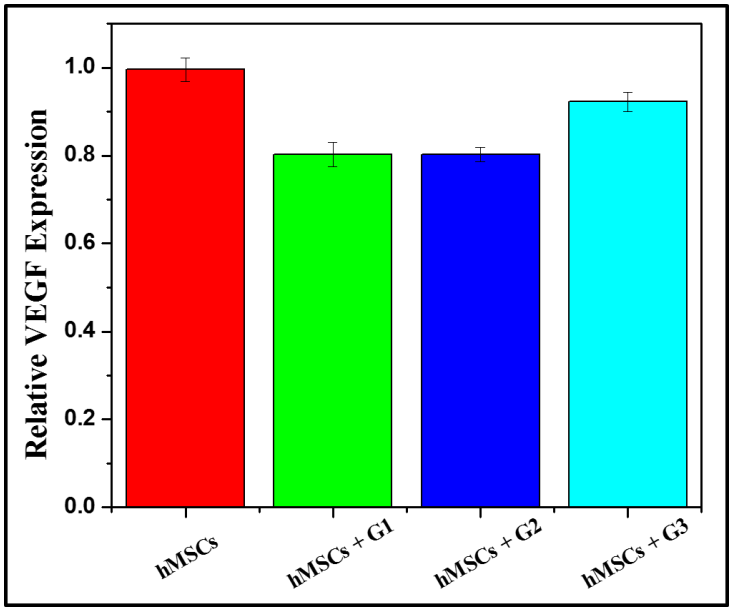

Figure 5.30: VEGF expression analysis of the the hMSCs cultured in the prescence of GPMV

Chapter 6

Conclusion

GPMV were synthesized from osteoblasts by using the working solution of paraformaldehyde/dithiothreitol. GPMV generated in the presence of blockers MβCD and Cyto-D notable difference was observed in the size and kinetics of GPMV generation. As cytochalasin-D inhibits the actin polymerization thus, inhibiting the membrane blebbing. MβCD is a cholesterol depleting agent is being proved by performing the biochemical assay and NMR spectroscopy. The fusion of GPMVs with hMSCs by using centrifugation based method is possible and is an efficient method to achieve the fusion. The analysis of fusion was done using confocal microscopy and flow cytometry. More than 15 % fusion efficiency was investigated by flow cytometry analysis. The quantitative osteogenic study showed that the alkaline phosphatase activity, total collagen synthesis and matrix deposition was significantly high in the hMSC fused with the GPMV i.e. hMSCs + G1, hMSCs + G2 and hMSCs + 3. This proves the hypothesis that the GPMVs can induce the osteogenic differentiation of the hMSCs. The RT-PCR study shows that the mRNA isolated for early and late osteogenic markers is significantly higher in the MSCs culture in the presence of GPMVs for both expansion and differentiation medium. All the assays performed proves the hypothesis that the GPMVs derived from the osteoblast can successfully fuse with the hMSCs and induce the osteogenic lineage of the hMSCs.

6.1 Future scope of the work

The pathway by which the GPMVs induces the osteogenic differentiation is still unknown. The study should be done find the factors by which the osteogenic lineage is induced in MSCs.

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