DEVELOPMENT OF MAGNETIC BASED MULTI-LAYER MICROPARTICLES FOR STEM CELL ISOLATION, ENRICHMENT AND DETACHMENT

by

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ABSTRACT

DEVELOPMENT OF MAGNETIC BASED MULTI-LAYER MICROPARTICLES FOR STEM CELL ISOLATION, ENRICHMENT AND DETACHMENT

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Stem cells have been studied and used in several cell-based therapies for regenerative medicine, 3D tissue-engineered scaffolds, and *in vitro* models for drug screening and testing. For instance, cell based therapy via the use of stem cells like Endothelial Progenitor Cells (EPCs) to treat patients with cardiovascular diseases, including ischemic heart disease, in-stent restenosis, and peripheral arterial occlusive disease. In addition, Circulating Cancer Cells (CSCs) have been used as an indication of cancer progression-free survival and for studying the mechanisms of tumor formation, metastasis, and anti-cancer drug screening. Due to their extensive diagnostic and therapeutic potential, various techniques have been developed to isolate and expand these stem cells. However, limitations associated with these methods include a low number of stem cells that can be isolated from blood and other sources, the use of harmful chemicals such as Ficoll and Trypsin, and the difficulty in their *ex vivo* expansion.

The objective of this research was therefore to develop magnetic-based multi-layer microparticles (MLMPs) that can (1) magnetically isolate stem cells without the use of Ficoll and harsh shear forces; (2) provide sequential release of proliferating and differentiating growth factors for stem cell enrichment; and (3) finally detach the cell in response to temperature

changes without the use of chemicals like Trypsin. MLMPs were formulated via layer-by-layer synthesis and characterized for their physicochemical properties. The MLMPs had core-shell structure with a spherical morphology of 50-80 µm, were successfully conjugated with antibodies for stem cell isolation, and provided sequential growth factor release profiles for cell proliferation and differentiation. Moreover, cells were successfully isolated (77% isolation efficiency) from a cell suspension and detached from particle surface (80% detachment in 30 min) by lowering the surrounding temperature below 32ºC. Specificity of isolation was demonstrated by isolating EPCs from a complex cell mixture. In addition, preliminary *ex vivo* studies with blood was performed to demonstrate the use of these particles for stem cell isolation. Lastly, cell enrichment profiles indicated steady cell growth with MLMPs in comparison to commercial Cytodex3 micro-carriers. All of the above mentioned results clearly indicate the potential use of MLMPs for stem cell isolation, expansion, and detachment with many benefits, highlighting the ease of isolation based on its magnetic property, isolation selectivity and enhanced enrichment owing to the gradual release of growth factors from the MLMP.

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CHAPTER 1

INTRODUCTION

1.1 Background

1.1.1 Stem cells and their potential

Stem cell research has embarked on an invigorating translational phase with the aim to achieve clinical solutions to address failed traditional attempts [1]. Scientific interest has diverted attention from gene and recombinant DNA therapy, to stem cell technology with a focus not only on the therapeutic aspect, but also on diagnosis and understanding of cellular functioning and development of novel therapeutics [2, 3]. Moreover, stem cell research is a rapidly expanding multi-disciplinary area and consists of an immense potential in the field of regenerative and restorative medicine. These multifunctional cells, with elaborate clinical functionality, have been deployed in several areas of research and clinical studies (Figure 1.1).

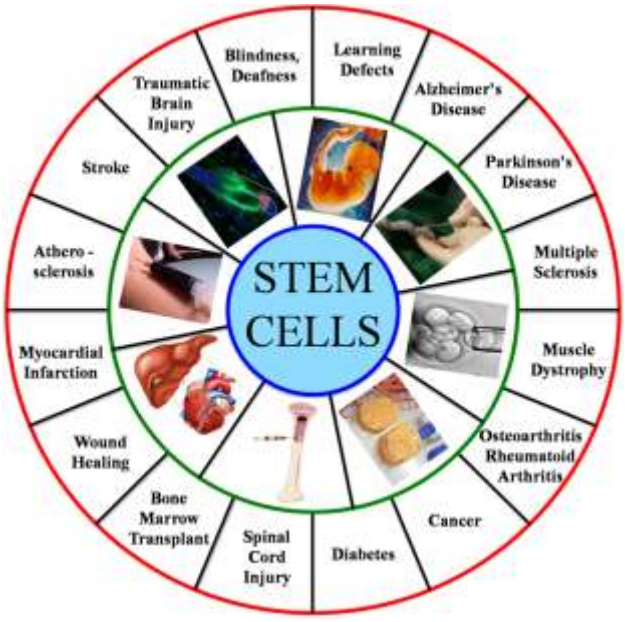

Figure 1.1 Pictorial depicting the sources and the immense potency of stem cells

Stem cell therapy/transplantation (SCT) aims at replacing damaged or diseased cells within the damaged tissue for treatments of various diseases. For instance, heart attack is proved to damage 25% of the left ventricle, which accounts for the loss of approximately one billion cardiomyocytes [4]. This condition thus accounts for the crucial requirement of an efficient stem cell number for transplantation to the damaged area for treatment. Human embryonic stem cell derived cardiomyocytes (hESC-CMs) have shown to improve myocardial function after transplantation into an *in vitro* ischemic heart model [5]. The potential of stem cells has not been limited to the research milieu but have extended a hand in clinical settings, highlighting the current employment of bone marrow transplantation to treat blood related disorders [1]. Another example of stem cell application is the use of limbal adult stem cells to restore retinal epithelium [6]. Stem cells also show immense potential as a model of spinal muscular atrophy, an early onset genetic disorder as well as non-genetic and late onset disorders [2]. Further, induced pluriopotent stem cells (iPSCs) have showed potential of differentiation into any desired cell lines, facilitating their use in tissue engineering applications and treatments of several disorders like Parkinson's disease.

Besides their use for treatment of various diseases, stem cells are also used for disease diagnosis (Figure 1.2). For example, breast cancer metastatic detection is performed by analyzing the presence of circulating tumor stem cells (CTSCs) in the peripheral blood by flow cytometry [7]. Cancer stem cells (CSCs) or CTSCs are clearly evident in chronic and acute myelogenous leukemia; efficient diagnosis of these CSCs in the hematopoietic system is crucial so that steps may be taken toward slowing down the disease progression rate [8]. In addition, Lian et al. outlined the potential benefits of iPSCs in making 3D models for drug screening and disease modeling, highlighting the fact that these cells manifest several advantages over other cell line studies [3, 9].

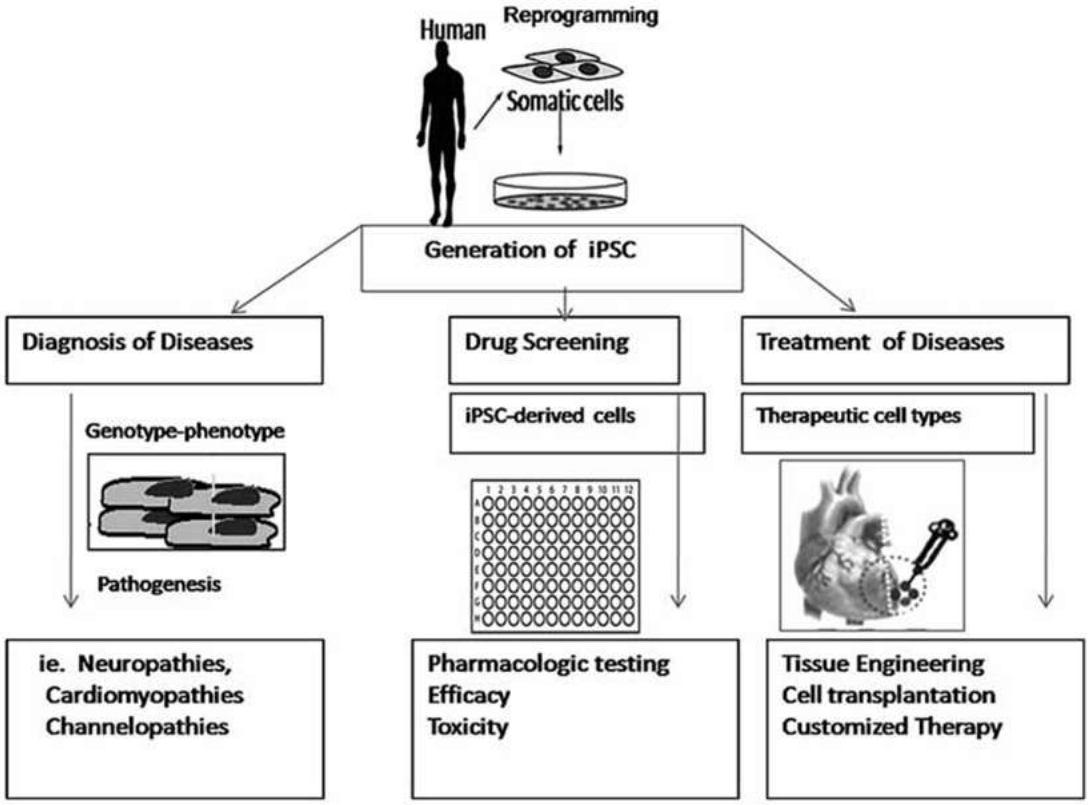

Figure 1.2 Therapeutic and diagnostic potential of stem cells [3].

Although stem cells can be isolated from various sources, including the bone marrow, embryo, peripheral blood, umbilical cord blood, adipose tissues, and specific organs/tissues such as the liver and skin, several limitations are associated with stem cell isolation. First, the number of stem cells in these sources may vary; for example, a significantly greater number can be obtained from cord blood in comparison to peripheral blood [10]. Some of the limitations of working with stem cells are the limited quantities that can be isolated from a patient; the small number of isolated cells that may not be sufficient to maintain a healthy live culture; and the possibility of alteration in stem cell fate due to environmental changes [11]. Taking into consideration the immense potential of stem cells in diagnosis and therapy, overcoming these limitations is needed so that stem cells can be used for diagnosis and therapy of various diseases.

1.1.2 Studied Stem Cells: EPC and LAPC4

Studies throughout this research project were performed with both Endothelial progenitor cells (EPCs) and the cancer stem cell line, Los Angeles Cancer stem cells (LAPC4). This section thus describes the details on both cell lines as well as their elaborate potential. Endothelial progenitor cells (EPCs) are bone marrow-derived progenitor cells that circulate in small fractions in the peripheral blood. EPCs are capable of self-renewal, proliferation and differentiation into mature endothelial cells (ECs). They do not yet express all the characteristic markers of mature ECs. Some of their typical characteristics are the presence of surface molecules like CD34, CD113, VEGFR-2, CD31, CD62E, CD144, vWF, migration and high proliferative capacity triggered by VEGF, exclusive uptake of acetylated LDL and binding to UEA [12, 13]. Phenotypical characteristics include spindle morphology after short-term culture of 7 days, changing into cobblestone growth after a prolonged period of 21 days. Proliferation of EPCs primarily depends on growth factor supplements, shear exposure, and topography of the growth surfaces [14].

In addition to the above characteristics, EPCs possess several major functions. For instance, EPCs contribute to tissue repair and neovasculature homeostasis by participating in angiogenesis and ateriogenesis. These cells are also capable of producing extracellular matrix that helps in improved scaffold formation and direct cell therapy in the area that requires treatment. In addition, EPCs have been known to repair the damaged endothelium as well as attenuate the progression of several cardiovascular based diseases such as atherosclerosis, myocardial infarction and stroke [12]. Differentiated EPCs into ECs have shown immense potential in management of neovascularization and cardiovascular disease including cardio ischemic conditions [15-17]. Due to their participation in various physiological and pathophysiological processes including arteriogenesis and angiogenesis, EPCs demonstrate their potential for coating vascular prosthetics such as vascular grafts and stents [14, 18, 19].

In addition to bone marrow, EPCs have multiple sources like peripheral blood, umbilical cord blood, and embryos. The adult bone marrow is considered the reservoir of these critical limited stem cells; the main reason behind this depleted number in circulating peripheral blood is the fact that they tend to mobilize from the bone marrow and into the blood. The cells in the circulating peripheral blood may also be termed as Circulating EPCs [19]. Recruitment of EPC from the bone marrow may be a result of the contribution of several factors like post vascular injury and ischemia, during tumor growth, granulocyte colony stimulating factor (G-CSF), proinflammatory cytokines, erythropoietin and apoptotic bodies from endothelial cells [12, 20, 21].

In order to isolate EPCs, the identified unique cell surface markers CD34, CD133 (or AC133), VEGF receptor 2 (VEGFR-2), and kinase domain receptor (KDR) have been used, and among these, CD34 is the most frequently used marker [22, 23].Mononuclear cells from blood contain EPC populations that are capable of differentiation into the endothelial cells, thus enabling efficient EPC isolation [24, 25]. Several techniques like Ficoll gradient centrifuge and CD34+ isolation kits have been developed to isolate and expand EPCs for further use. However, a major limitation with the use of EPCs in clinical applications is that their number in sources such as human blood is very low, accounting to an approximate count of 5×10^4 cells/ml [17, 26].In order to increase the number of these CD34+ cells, it may be preferable to grow them in the presence of a growth factor cocktail containing various essential growth factors and cytokines *in vitro* [10].

EPCs have been isolated and used in cell-based therapies for cardiovascular diseases. Infusion of EPCs has been demonstrated to improve neovascularization and heart functions for patients of various cardiovascular diseases and disorders such as coronary artery disease, acute myocardial infarction, pulmonary hypertension, and ischemic heart failure [19, 26-28]. In atherosclerotic conditions, cellular apoptosis results in the loss of vascular stem cells, thus impairing the regenerative capacity of the vascular wall [29]. Although the recruitment of these

stem cells towards the injured area is known to help in efficient repair of the vascular lining, the main limitations with a disease like atherosclerosis is that the capacity of stem cell survival, growth and differentiation of these stem cells is compromised by various atherogenic risk factors such as age, diabetes, inflammation and hypercholesteromia [4, 29]. EPCs may also be used as *indicators* for cardiovascular disorders, with the low EPC numbers indicating a higher risk of atherosclerosis [12]. A study done by Werner et al. concluded that a low number of EPCs may be used as a risk indicator for future cardiovascular based disorders [30, 31]. However, EPC quantification post cardiovascular event indicated a drastic increase as a result of ischemia [12]. Since the normal values of EPC in the blood are not standardized, the use of EPCs as a diagnostic marker may not be precise and accurate [13].

The potential for EPCs is not only limited to the cardiovascular field, but has also shown benefit in other diseases. For instance, EPCs have been used to treat *Cerebrovascular* diseases due to their capability of neovascularization in the presence of VEGF [4, 12]. EPCs may also be deployed in cancer therapy due to their ability to migrate, invade and thus incorporate in tumor sites [32].EPCs have been explored as therapeutic carriers that are capable of inducing controlled tumor cell apoptosis as homing of EPCs to the tumor site and is based on the tumor development characteristics, such as inflammation and the release of VEGF [33, 34]. The use of EPCs possesses many advantages not only based on their immense potential for cell based therapy and diagnosis, but also due to the fact that they do not pose any ethical concerns like those of embryo stem cells [12]. Highlighting the characteristics, potential, and advantages of EPCs, efficient and improved isolation techniques are a prerequisite for their utilization in therapy and diagnosis [14].

Circulating tumor cells (CTCs) and/or cancer stem cells (CSCs) have been shown to play an important role in the progression of various cancers, including prostate cancer. During cancer metastasis, cells leave the primary tumor, enter circulation, extravasate to a secondary

site, and form a new tumor. The cancer cells that travel to a secondary site are called circulating tumor cells (CTCs), whereas cell sub-populations that have the ability to self-renew, differentiate, and form tumors are called cancer stem cells (CSCs) [35]. CSCs and CTCs may or may not be same. They can be found in peripheral blood of patients during the early stage even when the primary tumor is not detected, by currently available methods [36]. These cells are generally resistant to conventional chemotherapy techniques and thus result in tumor relapse. CTCs shed from the primary tumor are found in low frequencies in the blood and thus posing a major challenge to identify and characterize them.

Due to their important role in tumor progression, CSCs/CTCs have been studied extensively and used in many applications. For instance, identifying CSCs/CTCs and characterizing their cellular origin, phenotype, and tumor-initiating mechanisms can help improve our knowledge and understanding about cancer biology, and in turn benefit towards the development of better cancer therapies [7]. Assessing treatment efficacy on these cells in individual patients is also important for establishing personalized medicine so that effective therapy may be administered while ineffective therapy can be discontinued [37]. The measurements of CSCs/CTCs levels in cancer patients before and during therapy as well as after the first follow-up visit have been performed on a number of cancer patients, and results show that CSCs/CTCs levels can serve as a useful indicator for tumor progression-free survival and overall survival [38]. Similarly, accessing efficacy of anticancer drugs and treatments is also essential while screening anticancer drugs and investigating novel anticancer treatments. Presently, the treatment efficacy is assessed by patient history and physical examinations, radiographic studies, and evaluation of serologic tumor markers. However, these approaches have certain levels of inaccuracy [39]. To improve the efficiency of CSC/CTC diagnosis, cellular analysis based on specific surface markers, such as CD117 for LAPC prostate cancer cells and CD44 for Ovarian cells, may provide an enhanced accuracy in diagnosis compared to that of traditional methods.

In addition to their use in assessing cancer therapy efficacy and establishing personalized medicine, CSCs have been used in *in vitro* 3D models for screening and testing of therapeutic reagents, as well as understanding the mechanisms involved with the development of drug resistance. Since tumor microenvironments are known to play a very important role in tumor progression and drug resistance, therapeutic resistance displayed by these rigid tumors pose a major obstacle in cancer treatment. Drug resistance may also be a result of secretion of cytokines and changing gene transcription that tend to override the cytotoxic effects of the cancer medication [40]. CSCs could be used in 3D *in vitro* culture models to investigate the effects of tumor microenvironments on the development of drug resistance in cancer cells, and to gain more knowledge about drug resistance in cancer. Thus it is important to isolate and expand CSCs for their immense potentials as mentioned earlier.

Some of the current CTC isolation techniques include the filtration approach based on increased sizes, microelectromechanical systems, antibody based capturing and microfluidic based chips. However, these methods lack sensitivity and reliability [35]. As a proof of concept, LAPC4 (Los Angles Prostate Cancer) cells have been used in our studies on cell isolation, owing to its metastatic characteristics. Isolation of these CSCs/CTCs is crucial for the diagnosis of metastatic cancers. These cells express CD117 surface markers and are known to grow in clusters with a more predefined rounded morphology. These metastatic cancer stem cells are capable of travelling via circulation and invading other organs of site by passage through microvasculature [41].

1.2 Current Methods in Cell Isolation

Several techniques have been explored in cell isolation, such as the use of chemicals like Ficoll, Fluorescence-activated cell sorting (FACS), cell isolation beads, and so on. All of these techniques have been in use for years and have shown some degree of success. However, these methods pose some limitations, for instance, the elaborate culture time, low

isolation efficiency, and the inability to obtain and grow the cells without injuring them. A few standard cell isolation techniques such as Ficoll gradient centrifugation, FACS, and commercial isolation beads from magnetic cell separation technology (MACS) as well as commercial beads Cytodex3 for cell expansion are discussed in the following section.

1.2.1 Chemical and centrifugation based techniques

Chemical based isolation techniques have been widely used for stem cell isolation. This technique is well established and straightforward. Briefly, a sample of blood is acquired from the patient and diluted with Ficoll, and the sample is centrifuged at 400g for 30 minutes at room temperature [42]. The mononuclear cell layer containing stem cells is then separated and washed with PBS to get rid of other undesired cellular populations like red blood cells (RBCs). The cells are then cultured over a time range on fibronectin treated surfaces to obtain a pure culture of EPCs [12]. The use of Ficoll and the several steps involving centrifugation may be harmful to these stem cells due to the exposure of chemicals and harsh sheer centrifugal forces [43]. Other limitations include labor intensiveness and sensitivity of technique, as slight modifications in procedure may result in the acquisition of cells other than true EPC populations [12]. Nevertheless, this EPC isolation technique is used extensively.

1.2.2 FACS (Fluorescence Activated Cell Sorting)

FACS makes use of the specific light scattering and fluorescent characteristics that are independent for specific cells. FACS is a specialized form of flow cytometry that has the ability to individually sort a complex heterogenous mixture of cells, based on their fluorescent light scattering properties. This technique makes use of a labeling agent like Alex fluro / FITC labeled antibodies that stain respective cells. Based on the amount of light emission and absorbance received, the cells are either counted or sorted from a complex mixture. Advantages include the purification of isolated cells, a short time requirement for sorting and capability of reading over

100,000 events per second [44]. However, large cell numbers are required for analysis and low cell throughput rate is one of the major disadvantages of FACS based sorting. Sterility of cell suspensions, requirement of skilled operators and the overall cost of the equipment are accounted as other drawbacks towards using FACS for cell isolation [45]. Another limitation is that the use of fluorescent-labeled antibodies for cell sorting might affect cell functions/behaviors for later use.

1.2.3 Separation by Polymeric / Magnetic Beads

Cell isolation and expansion by the use of polymeric/magnetic beads has grown over the past years, representing the primary step towards cell based therapy and *in vitro* 3D culture models[46]. A brief description of the most common and well-known beads such as Dynabeads, MACS, and Cytodex3are mentioned below.

Dynabeads from Invitrogen are commercially available superparamagnetic polystyrene beads having a diameter of 4.5 μm. These beads are coated with specific antibodies relevant to desired cell isolation. Isolation is performed by combining them with cell suspension at 2-8ºC within a mixer [47]. Cells are then isolated magnetically by the use of an external magnet. This isolation technique is efficient, owing to specific cell isolation based on antibody conjugation and magnetic property, but the limitations include the small size of the beads and the lack of surface for cell attachment and growth. Moreover, several buffers and solutions including solutions combining PBS-BSA and EDTA / trypsin are used in the process [48].

MACS Technology has provided an effective solution to specific cell isolation. These biodegradable particles are conjugated with specific antibodies and require specialized equipment and reagents to perform cell isolation. The CD34 MicroBead Kit is composed of 50 nm sized micro beads conjugated to monoclonal CD34 antibody, FcR Blocking Reagent that helps avoid unspecific cell labeling by Fc receptors, and MultiSort release reagent. Briefly, the mononuclear cells are isolated from whole blood by Ficoll-Paque and EDTA. The cell solution is

then diluted with FcR blocking reagent to inhibit unspecific binding, followed by the addition of CD34 MultiSort MicroBeads. After mixing for 30 minutes at 6-12ºC, the cells are washed within a LS+/RS+ column. The column is then placed in a separator and washed with a buffer to extract undesired cells. Once a desired fraction of cell purity is achieved, the magnetically labeled cells are incubated with MultiSort Release Reagent and then separated within a MS+/RS+ column to remove any remaining magnetically labeled cells. Finally, the stop reagent is added and the cells may be utilized as per their application [49]. Although this technique is widely used and has a high degree of cell isolation purity, it is associated with several limitations and disadvantages. Firstly, the isolation system involves the use of special equipment adding to the increased cost. Secondly, several reagents and buffers including Ficoll are involved in the various steps of isolation. Lastly, owing to the nano-size of the beads, there might be a chance for cellular uptake resulting in possible toxicity to the cells.

Collagen based microcarriers Cytodex3 developed by GE Healthcare have been used for studying the expansion and differentiation of anchorage dependent cells. These micro-carriers are composed of a thin layer of denatured collagen coupled to cross-linked dextran matrix and coated with gelatin over the surface. These micro beads, having an average diameter of 80 µm, are strong and rigid and capable of culturing various anchorage dependent cells like hepatocytes, endocrine cells, those possessing low plating efficiency, and sensitive cells. The major advantage of this technique is that they are transparent, enabling ease for microscopic examination as well as the efficacy in culturing cells that are generally difficult to culture *in vitro*. Owing to the biocompatible nature of the particle components, inherent cytocompatibily is achieved [50]. These beads have been used successfully in several research studies and for culturing various animal cell lines as wells as embryonic stem cells [51]. The limitation encountered is the need to use either proteolytic enzymes like trypsin and collagenases to detach cells from the bead surface, or dextranase to digest dextran, resulting in

a free cell suspension. Another major drawback is the unselective nature of cell anchorage and the difficulty in isolating the beads from a suspension [52].

1.3 Research Overview

Improved cell isolation techniques with high specificity and 3D cell culturing are essential for efficient cell separation and expansion. Conventionally, cells are harvested by the use of proteolytic enzymes such as trypsin and dispase that degrade extracellular matrix (ECM), cell adhesion molecules, as well as the receptor proteins expressed on the cell surface. Thus isolation and expansion of stem cells without the use of these chemicals seem more beneficial. In addition, isolation techniques with microbeads may provide surface for 3D attachment and subsequent cell growth. Cells grown in 3D cultures have shown improved gene expression profiles compared to that observed with 2D cell cultures [53]. In addition, 3D isolation and culture systems provide an increased surface area for attachment and growth compared to those of 2D culture systems.

1.3.1 Research Goal

The major goal of the research project was to synthesize microparticles that are capable of isolating specific stem cells and sustaining as a 3D cell culture. Rapid, label free, enzyme independent cell isolation is also the *key* to this research. Magnetic based multilayered microparticles were successfully synthesized, and their efficiency of cell isolation was successfully evaluated in this research.

1.3.2 Specific Aims

As previously mentioned, from the immense potential of stem cells for diagnosis and treatment, the major goal of this project is to synthesize magnetic based microparticles with the sole application to isolate specific stem cells from a complex cell suspension or a sample of

blood, expanse these cells to a sufficient number, and detach them without the use of chemical and physical factors. Two specific aims towards achieving this goal are:

- Aim 1: To synthesize temperature sensitive magnetic based microparticles and characterize them on the basis of size and cytotoxicity.
- Aim 2: To evaluate the efficiency of these MLMPs for isolation, enrichment, and detachment of specific stem cells.

1.3.3 Research Strategy

The design of these particles was intended to enable the ease of stem cell isolation. The proposed technique of cell isolation is simple and lacks intense procedures and particular skills. MLMPs were allowed to coalesce with a cell suspension within a rotatory bioreactor tube for a brief period of time at 37°C. The cell-particle complexes were then magnetically separated from the remaining suspension by an external magnet. These complexes were then cultured over time to enable cell proliferation and differentiation. Later, these cells may be detached from the particle surface by lowering the surrounding temperature to room temperature (<32°C).The cells can then be used for further applications. Schematic on the application of MLMPs for stem cell isolation, expansion, and detachment is as indicated in Figure 1.3 below.

Cell isolation by MLMPs is advantageous based on the following reasons; Increased isolation specificity by conjugation of highly specific monoclonal antibody over the particle surface, ease of cell separation based on its magnetic property, delivery of growth factors: VEGF for cell proliferation and bFGF for subsequent differentiation, and the facilitation of enzyme-free cell detachment with the help of temperature-responsive polymeric surfaces. MLMPs can thus be used in specific cell isolation, and the isolated cells can further be deployed in diagnosis of diseases like metastatic cancer, used in stem cell therapy, and applied for creating *in vitro* 3D models for testing / analysis.

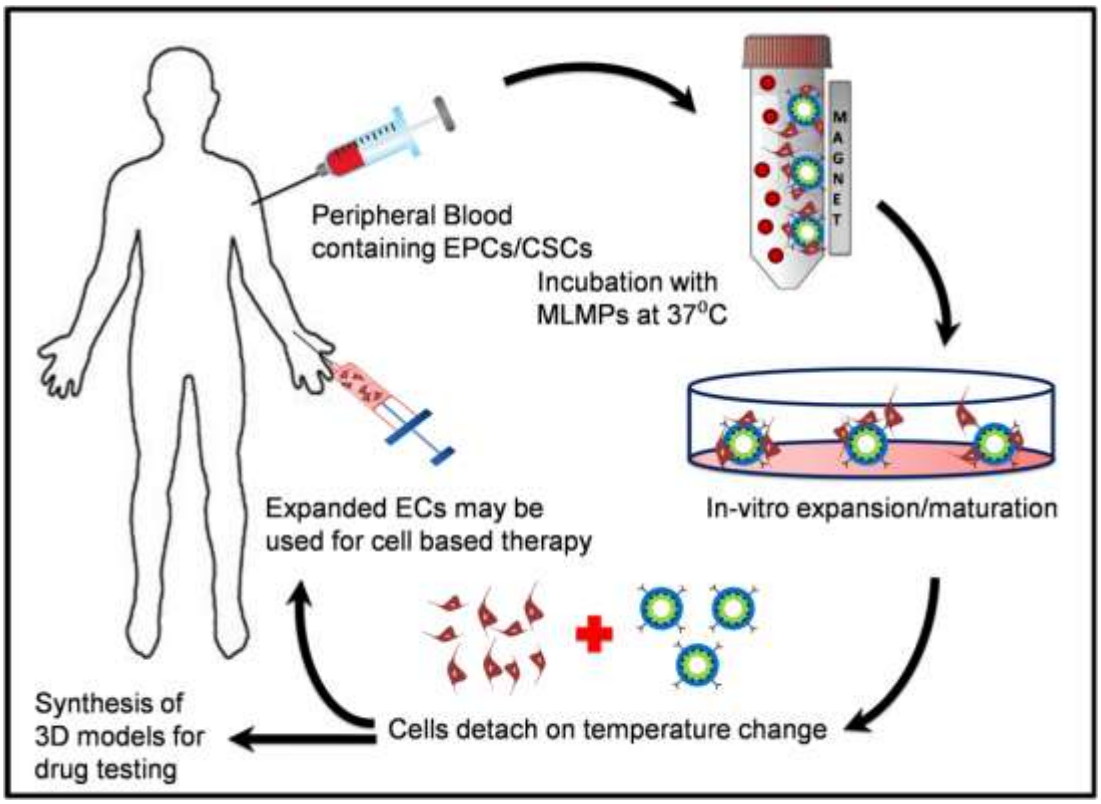

Figure 1.3 Schematic of application of MLMPs for stem cell isolation, expansion, and detachment.

CHAPTER 2

EXPERIMENTALS

2.1 Materials

Components required for the reactions included Poly (D, L lactide-co-glycolide) (PLGA, 50/50 with carboxyl end groups, Lakeshore Biomaterials) and Magnetic nanoparticles (Meliorum technologies). Other chemicals, including Dichloromethane (DCM), poly (vinyl alcohol) (PVA, 87-89%), *N*-isopropylacrylamide (NIPAAm, 97%),*N,N′*-Methylenebisacrylamide (BIS), Sodium dodecyl sulfate (SDS, 99%), potassium persulfate (KPS, 99+%), acetic acid, ethanol (95%), Allylamine(AH),Cytodex3microcarrier beads, Vinyltrimethoxysilane (VTMS, 98%), (3-Aminopropyl)triethoxysilane (APTMS, 99%),TEMED, N-(3-Dimethylamineopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), N-Hdroxy-succinimide (NHS, 98%), MES, were all purchased from Sigma-Aldrich.

For cell culture and *in vitro* studies, Endothelial basal medium (EBM-2) and SingleQuots Endothelial growth supplement (EGM-2) (Clonetics), 1X trypsin EDTA (Invitrogen), Iscove's Modified Dulbecco's Media (IMDM, Invitrogen), serum (Atlanta Biologics), penicillin-streptomycin (Invitrogen), Ficoll-Plaque PREMIUM (GE Healthcare), Dil-Ac-LDL (Biomedical Technologies), ELISA kit: VEGF, bFGF protein assay (Invitogen), Purified anti-human CD34, CD117 (Biolegend), Picogreen DNA assay (Invitrogen), Live / Dead Assay kit (Invitrogen) and 50 ml tube disposable bioreactors (Trasadingen, Switzerland) were used.

2.2 Polymer and Particle Synthesis

2.2.1 Synthesis of PNIPAAm-AH

The polymer was synthesized by free radical polymerization and characterized for its LCST and cell interaction properties. Polymer synthesis was performed by the following protocol: To prepare 10 ml of polymer solution, 0.06g NIPAAM and 60µl Allylamine was added to 10ml of DI water. After the NIPAAm has completely dissolved, BIS (0.021g) and APS (0.06g) that acts as a crosslinker and catalyst, respectively, were added to the solution. Lastly, 78µl of TEMED was added and the solution was purged with Agron gas. The reaction was stirred for 4-6 hours [54]. In order to get rid of all the un-reacted components and obtain a pure polymer solution, dialysis was performed using a 1000 Dalton dialysis bag by changing the dialysate 2-3 times per day.

2.2.2 MLMP Fabrication

MLMPs were synthesized by a step-by-step process that involved 3 major phases, i.e. synthesis of the PLGA microparticles, followed by coating with functionalized magnetic nanoparticles. The last step involved coating the particles with temperature sensitive polymer, PNIPAAm-AH. The schematic is as depicted in Figure 2.1 outlining the various layers of the particle and the growth factors loaded within them.

First, the PLGA microparticles were synthesized using a standard double emulsion solvent evaporation (W/O/W) technique [55]. The first water phase contains either water or water consisting of growth factors (ex. bFGF). 1ml of water / GF solution was added drop wise into 5ml of 2% w/v PLGA solution in dichloromethane (DCM). The resultant emulsion was finally added drop-wise to a 20 ml 0.5% w/v polyvinyl alcohol (PVA) solution and stirred at a speed of 450 rpm overnight to allow solvent evaporation [56, 57]. The double emulsion was then centrifuged at 1000 rpm for 5 minutes to collect the pellet. The pellet was then resuspended in

fresh DI water and centrifuged again. This washing step was repeated 2-3 times. The particles were then freeze-dried and stored at -20ºC for further use.

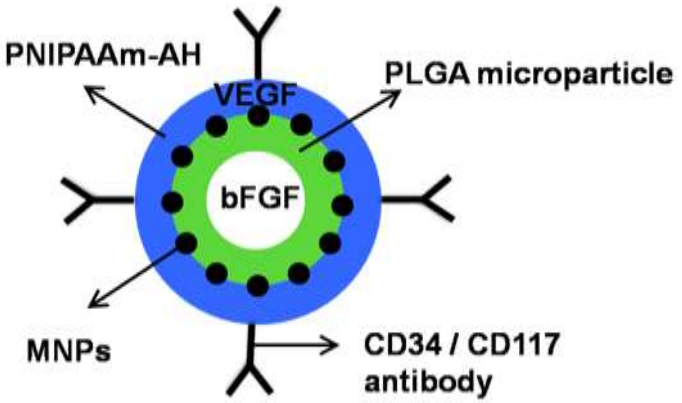

Figure 2.1 Schematic of the Multi-layered Magnetic based Microparticle (MLMP).

Prior to coating magnetic particles over the PLGA particles synthesized as described above, bare magnetic nanoparticles were functionalized with APTMS and VTMS. This was done so that necessary amine and silane groups are present over the magnetic particles, so as to achieve essential bonds for the attachment of MNPs over the PLGA particles as well as the temperature-sensitive polymer over the magnetic layer. APTMS provides amine groups for conjugation to PLGA microparticles by carbodiimide chemistry, while VTMS provides silane groups over these magnetic particles to enable links of PNIPAAm-AH onto PLGA-MNP particles via free radical polymerization.

For functionalization of MNPs with APTMS and VTMS, 0.07424 g bare magnetic particles were combined with 100ml ethanol in a polypropylene beaker; the solution was sonicated for 20 minutes at 3kW power with the addition of 3 ml acetic acid. The beaker was then placed onto a magnetic stirring plate, 243.5 µl of VTMS followed by 283.1 µl APTMS was added and stirred for 24 hours. The particles were magnetically collected, washed 2-3 times with ethanol and stored at 4ºC or used instantly in the next reaction [54, 55].

Coating of these functionalized magnetic particles over the PLGA microparticles is performed by carbodiimide chemistry in the presence of 0.1 M MES buffer. In brief, 20 mg of lyophilized PLGA microparticles was combined with 90 ml 0.1% w/v MES buffer and stirred for 20 minutes at 500 rpm. 0.16 g EDC is added to the flask and allowed to stir. Meanwhile, 14 mg of surface functionalized MNPs (synthesized as described above) were sonicated with 5 ml MES buffer in a separate beaker at 40 W. The solution was then added drop wise followed by the addition of 2mg SDS. The reaction is allowed to stir for 10 minutes and lastly 0.16g NHS is added to complete the carbodiimide reaction [54]. The reaction is stirred for 6 hours to allow conjugation. MNP-coated PLGA microparticles were then collected magnetically, washed twice with DI water to eliminate unused components including EDC and NHS. The particles were then freeze-dried before use.

The final step of MLMP synthesis involved copolymerization of PNIPAAm-AH over the PLGA-MNP particles. This free radical polymerization reaction involved the addition of 28 mg PLGA-MNPs to a conical flask with 90ml DI water and stirred for 30minutes at 500rpm. Then 0.3g NIPA monomer and 300µl Allylamine (AH) was added to the flask followed by 10.5 mg BIS and 1.5 mg SDS. The reaction is stirred vigorously for 10 minutes. Finally, 30mg APS and 39 µl TEMED was added simultaneously to the reaction and purged with argon gas. The reaction is allowed to take place for 4-6 hours [54, 58]. After the reaction, the particle suspension was centrifuged at 500 rpm for 5 minutes to pellet the particles, and the particles were then washed 2-3 times and freeze-dried to obtain the final product MLMPs.

Growth Factor Loading:

Two growth factors were loaded in the MLMPs, one within the innermost PLGA core and the other absorbed into the outermost PNIPAAm-AH layer. bFGF (Fibroblastic Growth Factor- basic) and VEGF (Vascular Endothelial Growth Factor) were used for our experiments. For bFGF, the growth factor was loaded during the initial double emulsion process replacing the

water used as W1 and mentioned above. bFGF solution (8ng/ml) was added drop wise into 5ml of 2% w/v PLGA solution containing dichloromethane (DCM). The resultant emulsion was added drop wise to a 20 ml 0.5 %w/v PVA solution and stirred at a speed overnight for solvent evaporation as described earlier. As for the second growth factor, freeze-dried MLMPs were added to the VEGF solution (8 ng/ml)in a tube and placed on a shaker at 4ºC for 3 days. This method of drug loading takes the advantage of the swelling characteristics of the temperature sensitive polymer [54]. The supernatants at both steps were collected to analyze their loading efficiencies indirectly as described previously [55].

2.2.3 Conjugation of Antibodies

MLMPs were conjugated with CD34/CD117 antibodies via Carbodiimide Chemistry. The conjugation was performed by the following procedure: 10 µg of antibody was added to 0.5 ml MES (0.1M) buffer along with 5mg EDC and allowed to combine for 30 minutes by gentle shaking. 5mg NHS and 1 mg MLMPs were added and allowed to react over 4 hours. The particles were then collected using a magnet and washed 2-3 times to remove non-conjugated antibodies as well as un-reacted chemical compounds. Fluorescently labeled antibodies were conjugated to the particles using the same protocol and analyzed for their binding efficiencies as described in the latter section on particle characterization.

2.3 Polymer and Particle Characterization

2.3.1 LCST Characterization for PNIPAAm-AH

Analysis of LCST characteristics of the polymer was performed by two methods namely (1) Visual Observation, and (2) Absorbance analysis by Spectrophotometry.

Visual Observation: 1 ml of synthesized PNIPAAm-AH solution (prepared as mentioned previously in section 2.2.1) was added to a transparent glass vial and placed onto a heating plate. The temperature was gradually raised and visual changes in polymer characteristics like

turbidity or cloudiness were observed. The temperature at which these changes were observed was noted as the LCST of the temperature sensitive polymer solution.

Absorbance analysis by UV-Vis Spectrophotometry: The absorbance values obtained from PNIPAAm-AH solution was measured by the UV-Vis spectrophotometer (Icontrol, M200) coupled with a temperature controller. Briefly, the absorbance of polymer solution (N=4) was analyzed at different temperatures ranging from 23ºC-39ºCwithin a 96 well plate. Absorbance readings were taken at a wavelength of 500 nm. The percentage transmittance was then calculated and plotted as a function of temperature. The LCST was determined by plotting the temperature corresponding to 50% transmittance[59].

2.3.2 Cell-Polymer Characterization

Cell Culture of EPCs and LAPC4 cells: Endothelial progenitor cells at passage 4-8 were used for all experiments. These sensitive stem cells were cultured in EBM-2 (Clonetics) and supplied with EGM-2 endothelial growth supplement (Clonetics).LosAngeles Prostate Cancer cells (LAPC4) was cultured in Iscove's media (IMDM, Sigma), while human dermal fibroblasts were grown in Dulbecco's eagle media (DMEM, Sigma). All cells were maintained in an incubator set at 5% CO_2 and at a temperature of 37ºC. The culture media was replaced every second day.

The ability of the MLMPs surface to support cell adhesion was tested by culturing EPC and LAPC4 cells on PNIPAAm-AH coated (glass) cover slips. The polymer was grafted onto the surface of glass cover slips and then sterilized before seeding cells, as described elsewhere by Tang et al [60]. The cells were seeded onto the coated cover slips and grown for 48 hours. EPCs were then immunostained with acetylated low-density lipoprotein labeled with the fluorochrome DiI (Dil-Ac-LDL) (Biomedical Technologies) and DAPI, while LAPC4 cells were immunostained with FITC-labeled antiCD117 and DAPI. Briefly, the cells on the cover slips were fixed using standard fixing solution (4% paraformaldehyde) and washed with PBS. EPCs

were incubated with 10 µg/ml Dil-Ac-LDL at 37°C for 4 hours and the excess stain was washed off with PBS. The nucleus was then stained with DAPI for about a minute in the dark. As for samples with LAPC4, the cells were fixed with paraformaldehyde and immunostained with FITC labeled CD117 by incubation for 1 hour. The cover slips were then washed with PBS and the nucleus was stained with DAPI. The cells were then imaged using fluorescence microscopy (Nikon) at 20x magnification. H&E staining of these cells by a standard protocol was also performed, and microscopic images were obtained. All the imaging was performed to compare their morphology, functionality and growth with the controls consisting of EPC / LAPC4 cells cultured directly on glass cover slips without PNIPAAm-AH coating.

A cytotoxicity study was also performed to quantify the biocompatibility of the polymer. In short, polymer was grafted onto the wells on a 96 well plate and sterilized in the presence of UV light. Cells were then seeded in the wells and a viability assay (MTS assay) was performed over a period of 48 hours following the manufacturer's instructions. Cells grown on the culture plates serve as a control.

2.3.3 Particle Characterization

The particles were characterized efficiently by several techniques, namely, (1) Dynamic light scattering (DLS) to analyze the size and polydispersity, (2) Scanning electron microscopy (SEM, Hitachi N3000) to visualize the particle/surface morphology, (3) Transmission electron microscopy (TEM) to visualize the presence of different layers within the particle, (4) Fourier transform Infrared spectroscopy (FTIR, Thermo FT-IR Nicolet-6700) to analyze the presence of various chemical bonds on the surface, (5) Vibrating sample magnetometer (VSM) to study magnetic properties, and (6) Bioconjugation of fluorescently labeled antibodies over MLMPs to determine the successful antibody conjugation. SEM, TEM and FTIR were performed at every step of particle synthesis. This was done to clearly differentiate and highlight the differences on

particle characteristics at subsequent stages of synthesis. All these tests were performed using standard protocols as described previously [54, 55, 61].

2.3.4 Growth Factor Release

MLMPs loaded with growth factors were analyzed for their release characteristics. Briefly, samples were incubated with PBS at 37ºC over a time range, and at a predetermined time point, the supernatant was collected and stored for further analysis. The release was performed over 14 days. The samples were analyzed using the VEGF/bFGF ELISA kits (Invitrogen) following the protocol as provided by the manufacturer. The percentage release was then plotted as a function of time. Growth factor loading efficiencies are also estimated as described earlier.

2.4 Cell Isolation and Detachment

MLMPs were used to isolate EPC/LAPC4 cells to emphasize on the primary application of selective cell isolation. MLMPs and experimental conditions were optimized to enhance cell isolation and detachment efficiency of the system. Studies with LAPC4 cells were done in order to verify the feasibility of MLMP usage for isolation of different cell lines only by modification of antibodies conjugated over the surface.

2.4.1 Optimization Studies

Quantitative analysis of isolated EPCs/LAPC4was performed in an extensive manner based on three independent factors including: (1) the amount of MLMPs used, (2) the incubation time, and (3) the amount of antibody conjugated over the MLMPs.

Firstly, the effects of incubation time (1, 2, 4 and 6 hours) on cell isolation were studied with 1 mg MLMPs conjugated with 5µg antibody and 100,000 cells. The time at which maximal cell isolation occurs is then selected for further optimization studies. Secondly, the effects of

MLMP amounts (0.2, 0.75, 1, 2 and 5 mg/ml) on cell isolation efficiencies were evaluated while keeping the number of cells (100,000) and antibody concentration (5 µg/mg MLMPs) constant. From this study, the concentration of MLMPs that isolated the most number of cells was chosen for later studies. Next, the effects of antibody amounts (1, 2, 3, 5, and 10 µg/mg MLMPs) on cell isolation efficiencies will be evaluated while keeping the number of cells (100,000) and concentration of MLMPs (from previous study) constant. Further, the concentration of antibodies that isolated the greatest number of LAPC4 cells was chosen for later studies. All experiments have a sample size of 8 (N=8).

In order to quantify the cells isolated for each sample, Picogreen DNA assay was performed and cross-referenced with that of a hemocytometer count. In brief, the cell-particle suspensions were incubated with lysis buffer (1X Triton) for 15 minutes. 100µl of lysate is then transferred in to a UV plate and diluted with 100 µl of Picogreen reagent. The fluorescence is read at ex-450 and em-520, the cell numbers are calculated based on a DNA standard curve[62, 63]. Direct analysis involved the quantification of DNA from cells detached from the particle surface, while indirect quantification was done on the obtained supernatant consisting of cells that were not isolated. Hemocytometer counting was also performed to confirm the results obtained by DNA assay analysis. All of the results were statistically analyzed using a two-tailed t-test and their p values were noted.

2.4.2 Characterization of the cell-particle complex

The cell-particle complexes were imaged using SEM and LIVE/DEAD assay (Invitrogen). Briefly, for SEM imaging the complexes were washed with PBS three times and fixed with 4% paraformaldehyde solution. Sequential ethanol dehydration was then done followed by lypholization with tert-butyl alcohol and osmium tetra oxide. SEM was performed with 2 kV voltage [64]. LIVE/DEAD assay was performed at 0.75 µM Calcein and 0.25 µM EdtH-1 concentration for particle-cell complexes with EPCs[65]. The dye was added and incubated at

37ºC for 40 minutes. The samples were washed with PBS and imaged at 20X magnification with the appropriate filters to detect live and dead cell populations on the particles.

2.4.3 Cell Detachment

The effects of time (30, 60 and 120 minutes) on cell detachment (after placing cell-particle complexes at room temperature) were evaluated while keeping concentration of MLMPs and CD117 antibodies (from previous optimization study) constant. From this study, the detachment time at which the number of detached cells was saturated will be chosen as optimum detachment time. Briefly, the complexes are separated and removed from the bioreactor; they are introduced within wells of a 48well-plate and allowed to incubate at room temperature to allow cell detachment from the MLMP surface. The detached cells at various time points were quantified using DNA assays as described earlier. After detachment, cells were also further cultured on a glass slide and incubated at 37ºC for 12 hours to allow cell adhesion and growth. These samples were viewed under microscope observation and compared with normal cultures.

2.4.4 Cell Isolation from a Mixture of Cells

Cell isolation from a mixture of cells was performed to prove the selective isolation efficacy of the system. This was done by mixing two cell types, thus forming a complex mixture and then performing the isolation study based on the optimized values of concentrations and time as studied from the experiments described in the previous sections. 100,000 cells of each cell line (HDFs and EPCs) were mixed together within a bioreactor tube and combined with 1 mg antibody conjugated MLMPs. Isolation conditions were maintained as per the optimized values. Cell-particle complexes were then separated using an external magnet. The cells were then detached and analyzed by immunostaining with FITC labeled CD34, DAPI and Dil-Ac-LDL.

2.5 Blood Studies

To test the cell isolation efficacy of our MLMPs, cell isolation studies were performed and compared with traditional Ficoll density gradient procedure. All studies with human blood were performed according to the regulatory outlines of stem cell research and the University medical services (UTA IRB board, 2008.569S), keeping in consideration that translational stem cell based therapy must be accompanied by the development of regulatory oversight of basic research with translational potential [1, 66].

2.5.1 Isolation of EPCs using Ficoll-Paque PREMIUM

Our system was compared against the traditionally used stem cell isolation technique, Ficoll-Paque gradient centrifuge. The technique is simple and well established. In brief, a sample part of human peripheral blood was combined with a Ficoll in the ratio of 5:3. The resultant solution was then centrifuged at room temperature for 35 minutes and 400g. Following centrifugation, four layers were obtained namely; plasma with platelets, mononuclear cells containing stem cells, Ficoll and lastly red blood cells; from top to bottom. The mononuclear cell layer is then extracted and washed twice with PBS by centrifugation at 1000 rpm for 5 minutes[12, 23, 67]. This step must be done with precision (gently) to minimize the mixing of different layers. The cells were finally cultured in fibronectin-coated flasks over a time course and imaged for morphology and proliferation changes.

2.5.2 Isolation of EPCs using MLMPs

To isolate EPCs from blood, 1mg of anti-CD34-conjugated MLMPs were added to 5 ml blood within a bioreactor tube and set for rotation at 37 ºC. Post isolation, the cell-particle complexes were separated via magnet, and these complexes were cultured in conditioned media over a period of 14 days. The cells were compared with that isolated by Ficoll.

2.6 Cell Expansion Study

Cell expansion using MLMPs was studied and compared with commercially available Cytodex3 microbeads purchased from Sigma-Aldrich. Briefly, the study was done over 3 weeks and 5 different groups were considered. The groups included EPCs cultured over (1) Growth factor loaded MLMPs (incubated with growth factor deprived media), (2) Unloaded MLMPs and media supplemented with growth factors, (3) Unloaded MLMPs and growth factor deprived media, (4) Cytodex3 and media supplemented with growth factor, and (5) Cytodex3 with growth factor deprived media (N=4). In all these studies, EPCs were cultured at 5% CO_2 and 37°C. First, the rotation study was performed as mentioned above, on the basis of optimized results. The particles were separated from the suspension to obtain the particle-cell complexes and the supernatant was analyzed to quantify the initial isolation efficiency. The cell-particle complexes were cultured over a time range, and the media was changed every 3 days. The cells grown on the micro-particles after specific days of cultivation were detached from the surface, and a DNA assay was performed to quantify the total cell DNA. DNA assay was performed by a standard protocol as mentioned above and cell growth curves were plotted. Statistical analysis was performed and their p-values were obtained.

CHAPTER 3

RESULTS

3.1 Polymer Characterization

PNIPAAm-AH was characterized for its LCST, biocompatibility, and cell interaction characteristics, and the results are presented in the next section.

3.1.1 LCST of PNIPAAm-AH

The LCST of PNIPAAm-AH was determined by visual observation and UV-Vis spectrophotometer. The plot for percentage transmittance versus temperature is shown in Figure 3.1. Results from spectrophotometer analysis indicated an LCST observed at 33°C when transmittance reduced to 50%. While in visual observation, the solution started turning cloudy at 33°C (right tube), whereas a clear solution was seen at room temperature 25^0C (left tube).

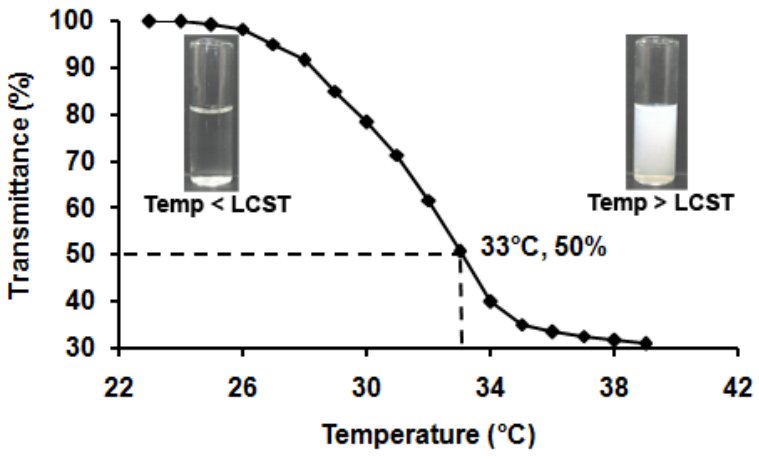

Figure 3.1 LCST characteristics of PNIPAAm-AH by spectrophotometry and visual observation.

The varying absorbance values observed at different temperatures are due to the increasing turbidity caused by polymer shrinking at higher temperatures. At temperatures below LCST, less absorbance of light is observed. Thus, the particle surface covered with PNIPAAm-AH polymer is hydrophobic at incubation temperatures of 37°C or above, and hydrophilic at temperatures below LCST (below 33°C).

3.1.2 Cell-polymer Characterization

The polymer was characterized for its biocompatibility on the basis of cell viability on the polymer surface over time. As seen in Figure 3.2 below, cell viability remained high with about 80% cell viability after 48 hours of exposure to the polymer. There was no significant difference seen in cell viability over the time range between the control and polymer groups.

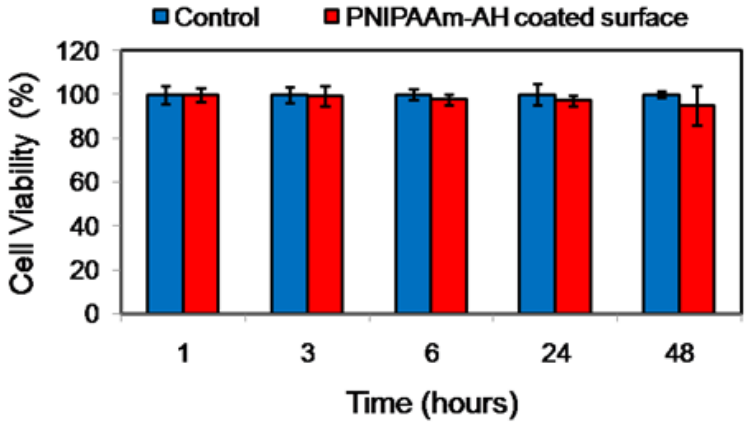

Figure 3.2 Cell viability analysis of PNIPAAm-AH coated and control surfaces over 48 hours.

Immunostaining studies of EPCs were performed with Dil-Ac-LDL and DAPI. Figure 3.3 indicates the cytoplasm stained with DIL-Ac-LDL and the nucleus stained with DAPI. Dil-Ac-LDL is known for cytoplasmic staining of EPCs by liposomal uptake of DIL, while DAPI is a commonly used stain to view the nuclei. The fluorescence images were taken individually and superimposed. Morphology and cell viability over polymer surfaces were observed and

compared with that on control slides, displaying no significant morphologic differences. H&E staining was also performed to validate the fact that PNIPAAm-AH does not affect stem cell morphology, attachment, and growth.

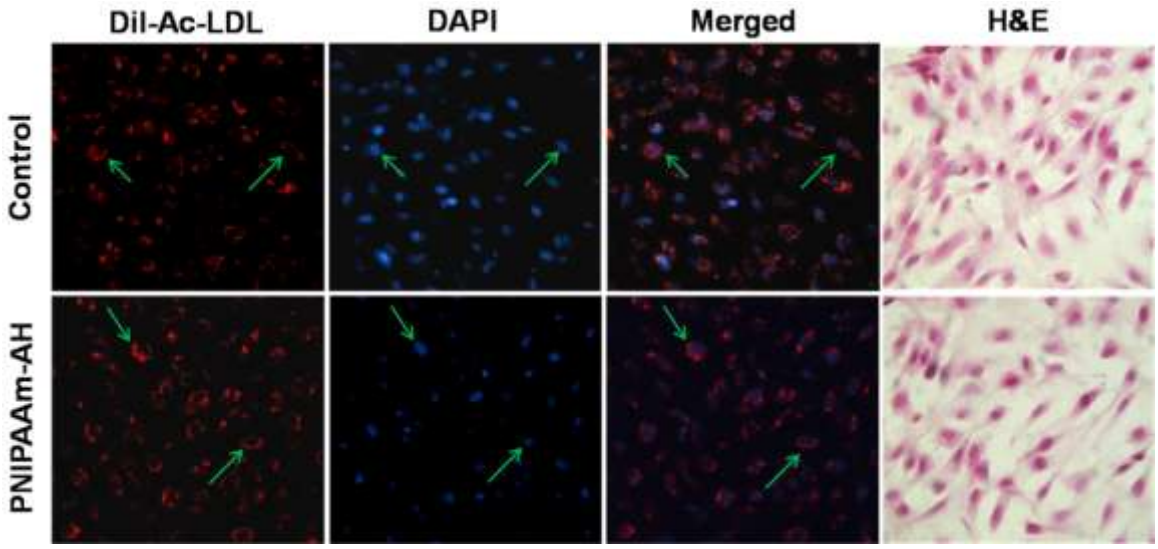

Figure 3.3 Immunostaining of Endothelial Progenitor Cells (EPC): Cell nuclei stained with DAPI (blue) and cytoplasm stained with Dil-Ac-LDL (Red). H&E staining of cells on polymer and control substrates.

Similar observations were obtained after immunostaining was performed on LAPC4 cells cultured on PNIPAAm-AH coated glass cover slips. Figure 3.4indicates the cells stained with both FITC-tagged CD117 antibody and DAPI as well as with H&E. The cells were healthy and attached to the polymer surface while maintaining their morphology on PNIPAAm-AH coated cover slips as compared those of the control group. This indicates that the particle surface is hydrophobic at the incubation temperature of 37°C and supports cell adhesion and growth.

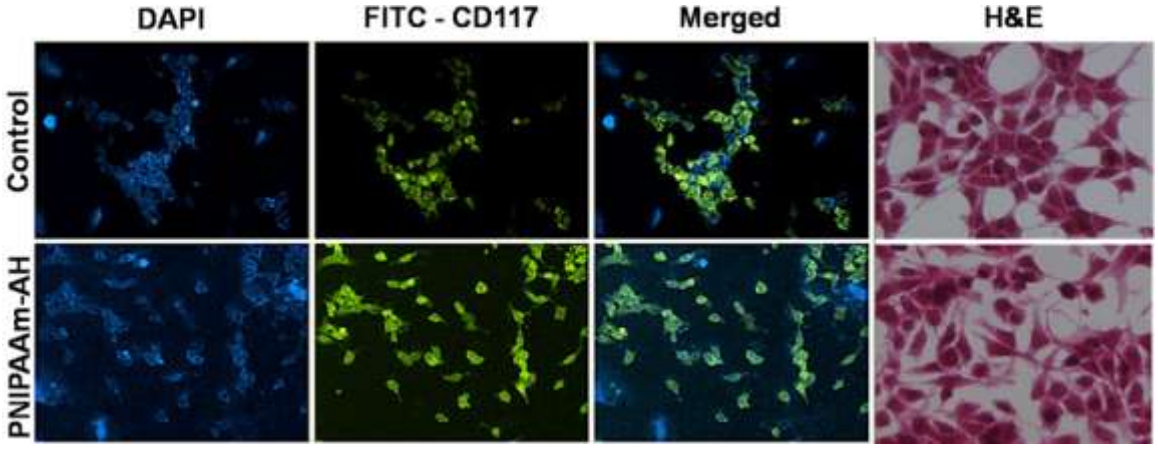

Figure 3.4 Immunostaining of LAPC4 cells. Cell nuclei are stained with DAPI (blue) and CD117 markers are labeled with FITC-labeled CD117 antibodies (green). LAPC4 cells observed after H&E staining showing their adhesion and morphology.

3.2 Particle Characterization

3.2.1 Physiochemical Analysis of MLMPs

MLMPs were characterized at each step of synthesis for its surface morphology, particle diameter and chemical composition. The methods of characterization used were Scanning Electron Microscopy, Transmission Emission Microscopy, Dynamic Light Scattering, FTIR, VSM and Fluorescent label antibody conjugation. Results of each technique are described in detail in the following sections.

The MLMPs had a core-shell structure with spherical morphology as determined by TEM (Figure 3.5 a) and SEM (Figure 3.5 d), respectively. The particle size varied from 50-100 µm. SEM of PLGA microparticles (Figure 3.5 B) shows a very smooth surface, which became rougher in each step after conjugating MNPs on the surface of PLGA microparticles (Figure 3.5 C) and polymerizing PNIPAAm-AH on the surface of MNPs-conjugated PLGA microparticles (Figure 3.5 D).

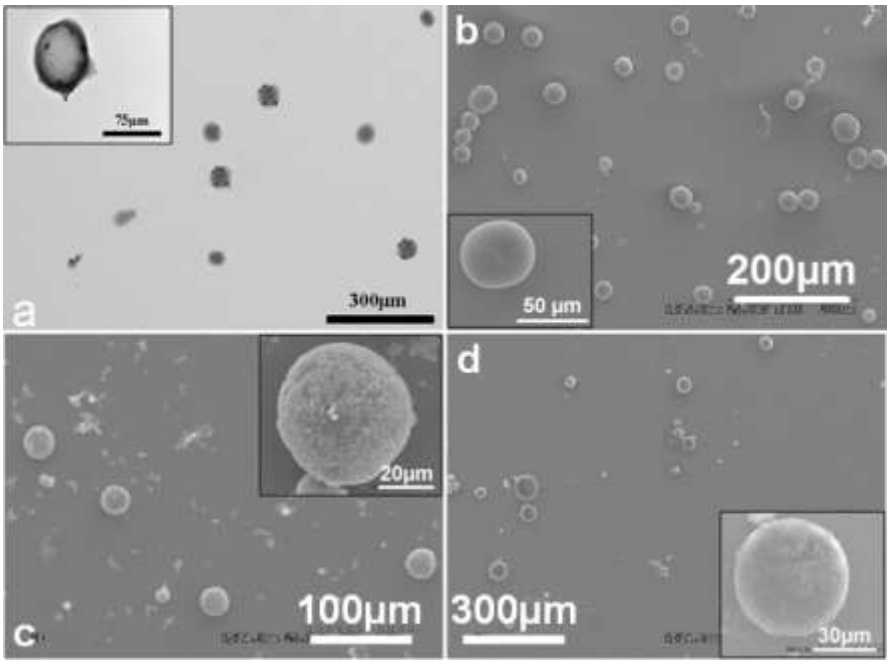

Figure 3.5 Physical characterizations of microparticles. (A) TEM image of MLMPs; SEM image of (B) PLGA microparticles, (C) MNPs-conjugated PLGA microparticles, and (D) MLMPs.

The successful polymerization of NIPAAm and AH monomers onto the MLMPs was confirmed via FTIR. As shown in Figure 3.6, FTIR spectrum of MLMPs was compared with that of PLGA microparticles and MNPs-conjugated PLGA microparticles to confirm the presence of PNIPAAm-AH on the MLMPs surface. The vinyl bonds (700-800 cm^{-1}) on silane-coated MNPs disappeared in FTIR spectrum of MLMPs. The –CH– stretching vibration (2936-2969 cm^{-1}) of the polymer backbone and two peaks in between 1600-1750 cm^{-1} correspond to the bending frequency of the amide N-H group and amide carbonyl group, respectively, which confirms the presence of amine corresponding to the AH. From these observations, it can be confirmed that PNIPAAm-AH has been successfully coated onto the surface of the PLGA-MNPs microparticles.

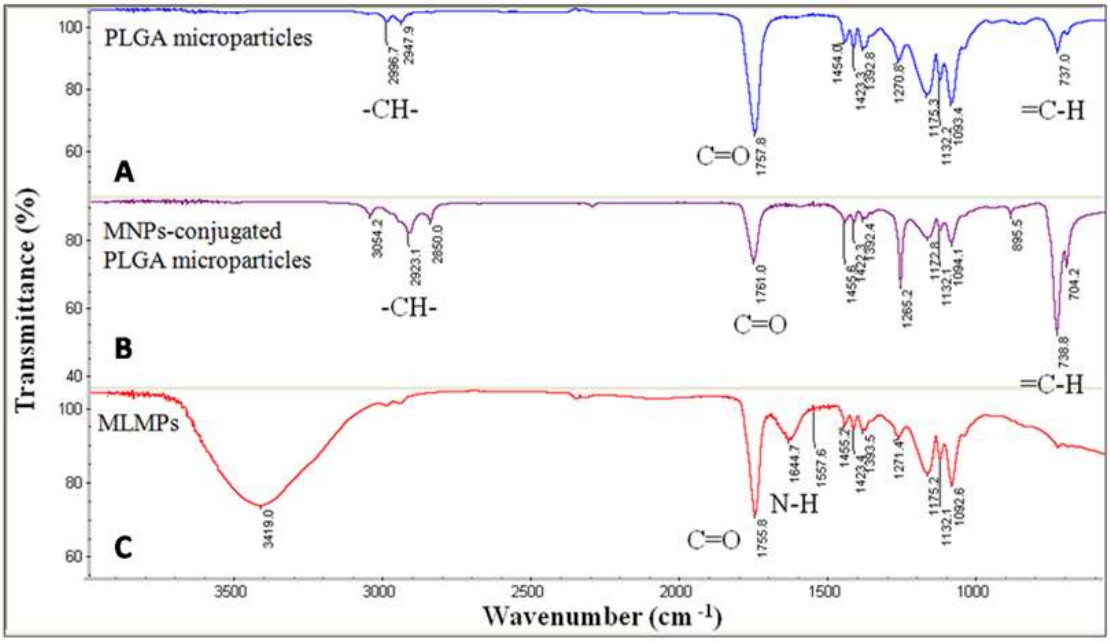

Figure 3.6 FTIR spectra of (A) PLGA microparticles, (B) MNPs-conjugated over PLGA microparticles, and (C) MLMPs.

Table 3.1Particle characterizations for average diameter and zeta potential using Dynamic light scattering (DLS), and Iron content values.

Particles	Average Diameter *(μm)*	Zeta Potential *(mV)*	Iron Content *(mg/mg particle)*
PLGA	41.29	-28.03	0
PLGA-MNPs	53.30	-13.02	0.44
MLMPs	83.26	-6.93	0.18

Dynamic light scattering analysis for diameter and zeta potential was performed at every stage of particle synthesis. Increasing diameter and zeta values were observed after

adding each layer. Increasing diameter from 41.29 nm to 83.26 nm for MLMPs indicates the success of coatings, while surface charges increased from -28.03 mV to -6.93 mV (Table 3.1). An iron assay was also performed to determine the iron content within the particles. 0.44 mg iron was presented in 1 mg of PLGA-MNPs, whereas 0.18 mg MNPs were in 1 mg of MLMPs.

Figure 3.7 below indicates the magnetic properties displayed by the MLMPs. On placing a 1.3 Tesla magnet against a vial of well-dispersed particles, attraction of MLMPs toward the orientation of the magnet takes place resulting in a clear solution (left). Using VSM, the hysteresis loops (right) was obtained and compared with that of bare magnetic particles providing values of -20 and -60, respectively. This indicates that the MLMPs possess a suitable magnetic property capable of magnetic separation with an external magnet even though their magnetic properties are reduced compared to that of original MNPs.

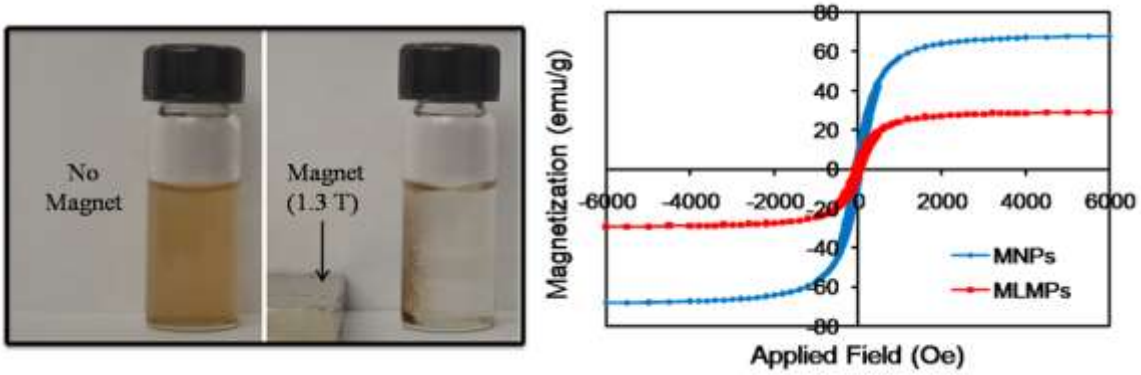

Figure 3.7 Magnetic properties of MLMPs by Visual observation using a 1.3 T magnet (left), and Hysteresis loops obtained by Vibrating sample magnetometer (VSM) comparing properties of MLMPs with bare magnetic nanoparticles (right).

3.2.2 Conjugation with Fluorescence labeled Antibodies

To confirm the conjugation of antibodies over the surface of the MLMPs, we conjugated particles with fluorescently labeled antibodies, including FITC-labeled CD34, Alexa Fluor 647-labeled CD34, and FITC-labeled CD117. The fluorescence from the particles was imaged using a fluorescence microscope (NIKON).

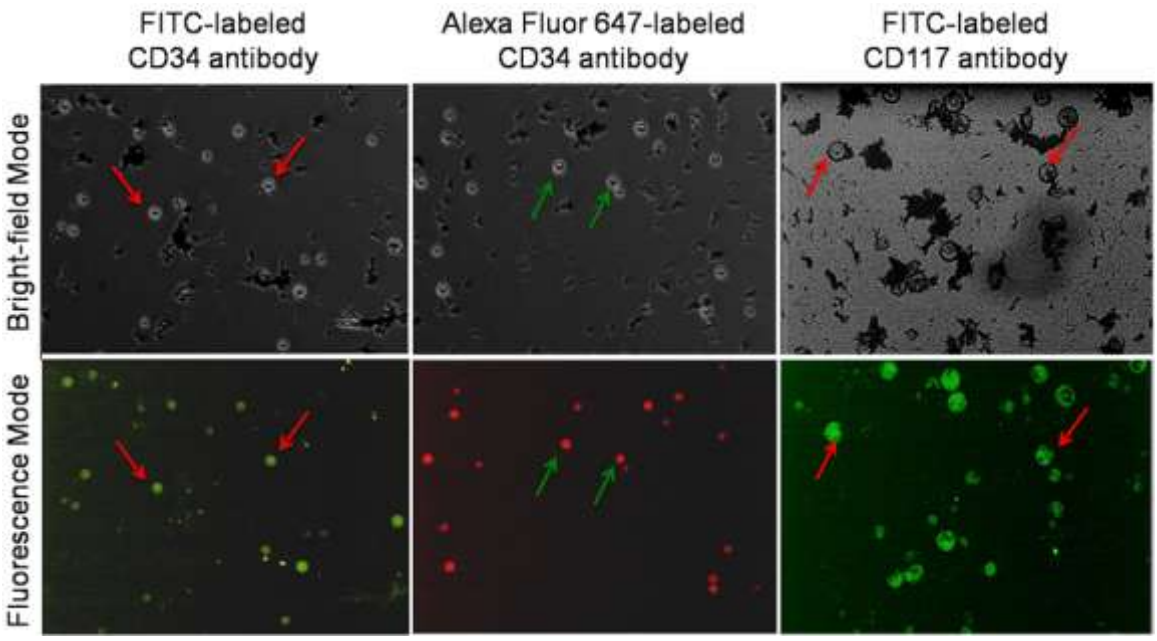

Figure 3.8 FITC and Alexa Fluor 647 labeled CD34 / CD117 antibodies conjugated over MLMPs depicting the successful conjugation of antibodies over particle surface.

Figure 3.8 shows images of fluorescently labeled particles compared with that at monochrome settings. A bright green color from FITC-labeled CD34 / CD117 antibodies and a bright red color from Alexa Fluor were observed on the MLMPs surface in the fluorescence mode compared to no fluorescence in monochrome mode. These observations suggest successful conjugation of CD34 / CD117 antibodies to the MLMPs. Successful conjugations of antibodies over the particles also confirmed the presence of various chemical bonds over the microparticles, as established by FTIR results presented in Figure 3.6.

3.2.3 Growth Factor Release Profiles

The release profiles of growth factors loaded within the core and the shell; bFGF and VEGF, were evaluated over a period of 14 days. As shown in Figure 3.9, for both the growth factors, an initial burst release was observed followed by a sustained release. The maximal growth factor release of VEGF from the shell was sustained at around 60% at 14 days, while that of bFGF from the core was around 40%. Analyzing the initial time points of growth factor release, at 5 hours a release of 20% was observed for VEGF, while there was a 7% release of bFGF from the core. By 12 hours, the percent VEGF release steepened to 32%, while that of bFGF remained at a meager 12%. This lag phase observed from bFGF is essential so that this differentiation growth factor is available at latter time points for cell differentiation.

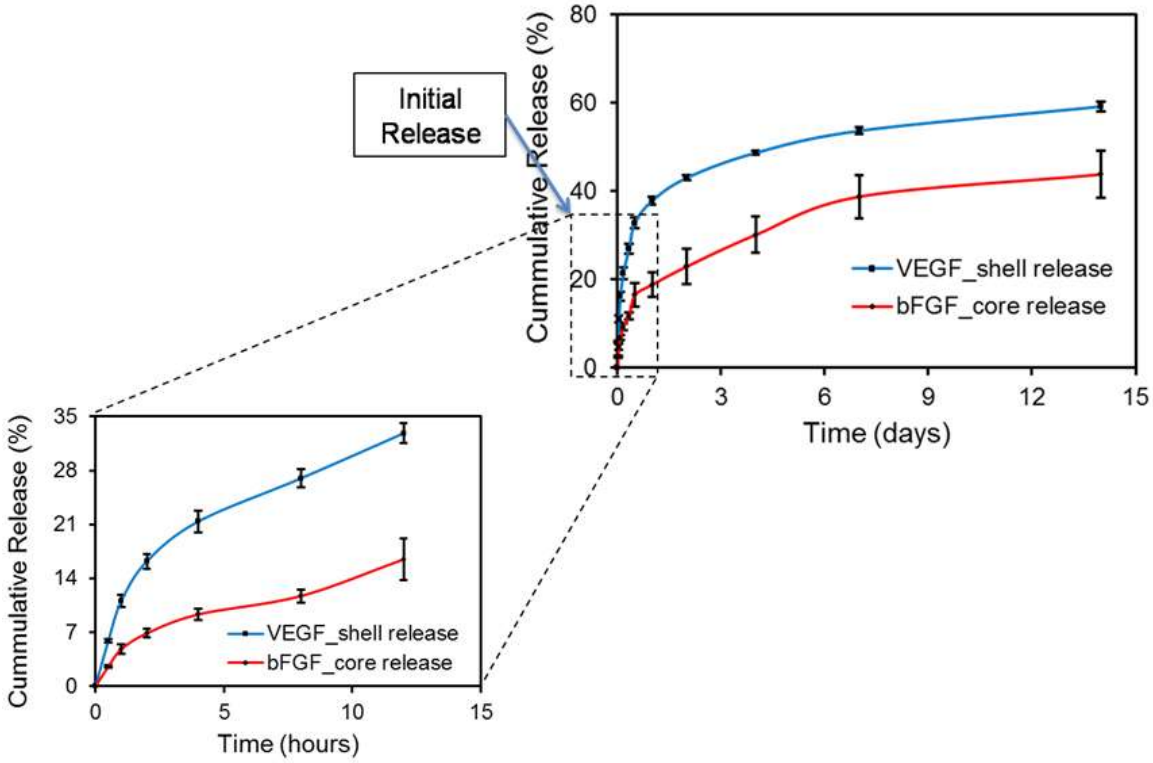

Figure 3.9 Growth factor release profiles of VEGF and bFGF (for proliferation and differentiation) from the shell and core, respectively over 14 days. Initial release time points (enlarged) indicating lag phase from differentiation growth factor encapsulated in the core.

3.3 Cell Isolation and Detachment Optimization

In order to quantify the degree of cell isolation and the amount of particles needed to isolate cells with the highest efficiency; various parameters were optimized with respect to cell isolation from pure cell cultures. The parameters studied include time, particle concentration, and antibody concentration conjugated over the particles.

3.3.1 Effects of time, MLMP concentration, Antibody concentration

The graphs below in Figure 3.10 indicate the optimization results with respect to various parameters: time, MLMP concentration, and antibody concentration. Firstly, results obtained by optimization of time indicated a high isolation efficiency of 77.4% after 2 hours (Figure 3.10 A) as compared to the values obtained at 1, 4 and 6 hours. The p values obtained after statistical analysis comparing results obtained at 1h and 2h were 0.005 and 0.035 for DNA Assay and hemocytometer count, respectively. Secondly, the optimal isolation efficiency was 74.3% with 1 mg MLMPs as indicated in Figure 3.10 B while varying concentration of MLMPs keeping the time constant as 2 hours (no significant difference). Finally, an isolation efficiency of 69.05% (p-values 0.021 and 0.046) was obtained with 1mg particles conjugated with 3 µg/mg antibody. In all, time was optimized as 2 hours, concentration of MLMPs as 1 mg/ml, and antibody concentration as 3 µg/mg. These optimized values were used for all further studies. Hematocytometer reading also revealed a similar trend compared to that obtained from the Picogreen DNA assay.

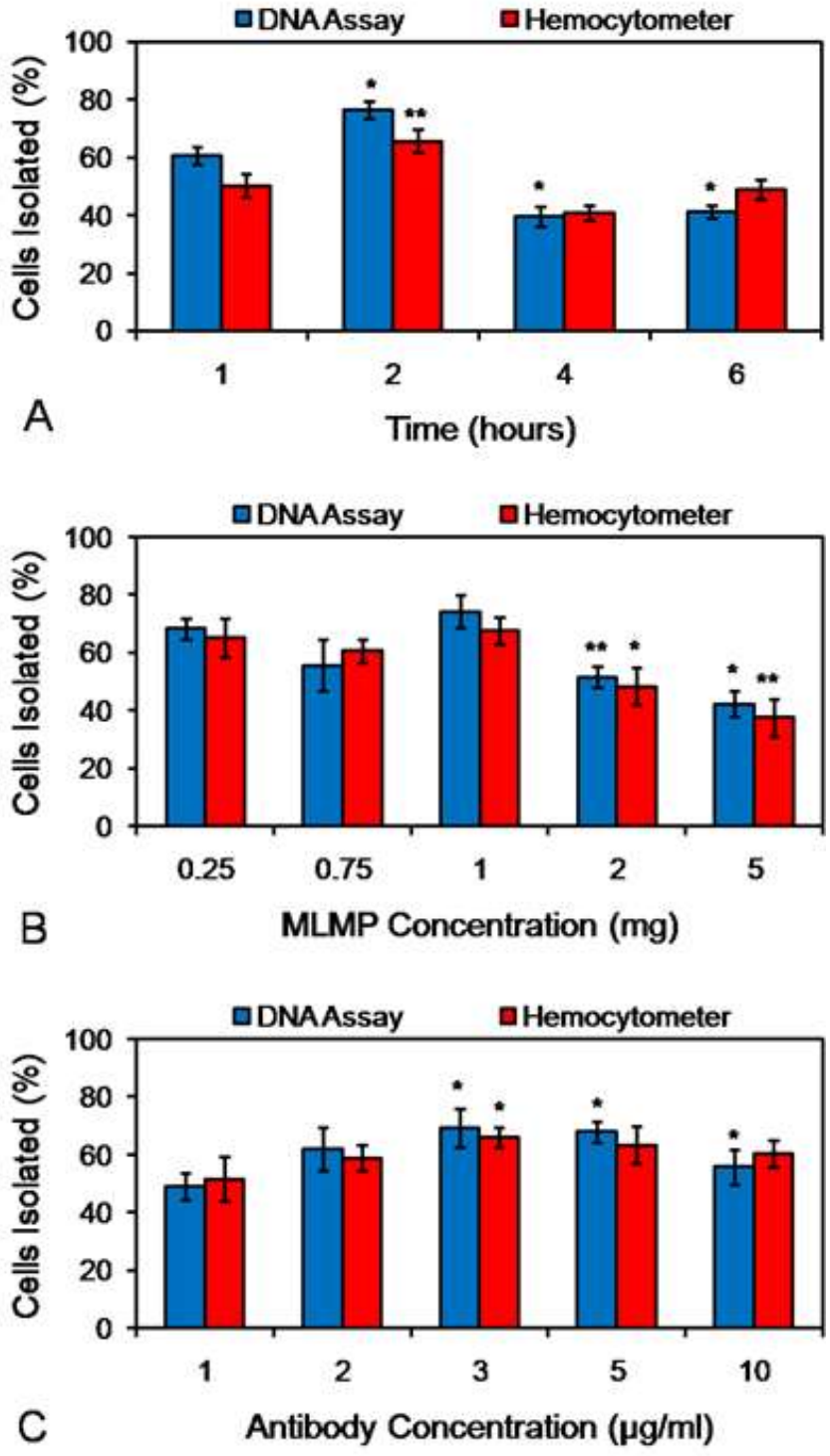

Figure 3.10 Cell isolation optimization (A) based on time in hours, (B) based on MLMP concentration used and (C) based on the amount of antibodies conjugated over the particle surface (*p<0.05).

3.3.2 Cell Detachment from MLMPs

For optimizing cell detachment, a detached percentage as high as 80% was observed during the first 30 minutes after lowering the surrounding temperature (room temperature) and the detachment values remained at saturation during 60 and 120 minutes as seen in Figure 3.11. No significant differences were observed among all the three time points ($p>0.05$).

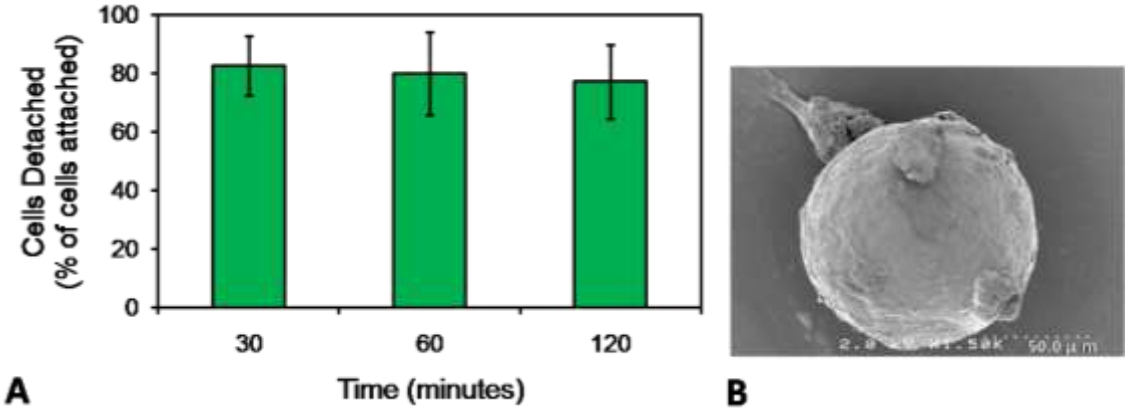

Figure 3.11 Cell Detachment (A) Graph plotted for detachment efficiency as a function of time for cell detachment from MLMP surface. (B) SEM image of Cell-particle complex.

Figure 3.11 B shows the SEM image of only a few cells attached to the particle surface after detachment. LIVE/DEAD assay and microscopic images of cells detached are shown below in Figure 3.12, indicating maximal cell detachment and minimal attachment over the particle surface. Figure 3.12 A is the monochrome image while Figure 3.12 B indicates the fluorescence image obtained by LIVE/DEAD staining. The particles are outlined for the purpose of clarity. Most cells are alive (green), and no dead cell (red) was observed. Figure 3.12 C and D show microscopic images of cell detachment at 30 and 60 minutes, respectively. Further, the detached cells were cultured in culture flasks and grown over time. No morphology differences were observed as in Figure 3.12 E and F for cell cultures after 7 and 21 days, respectively.

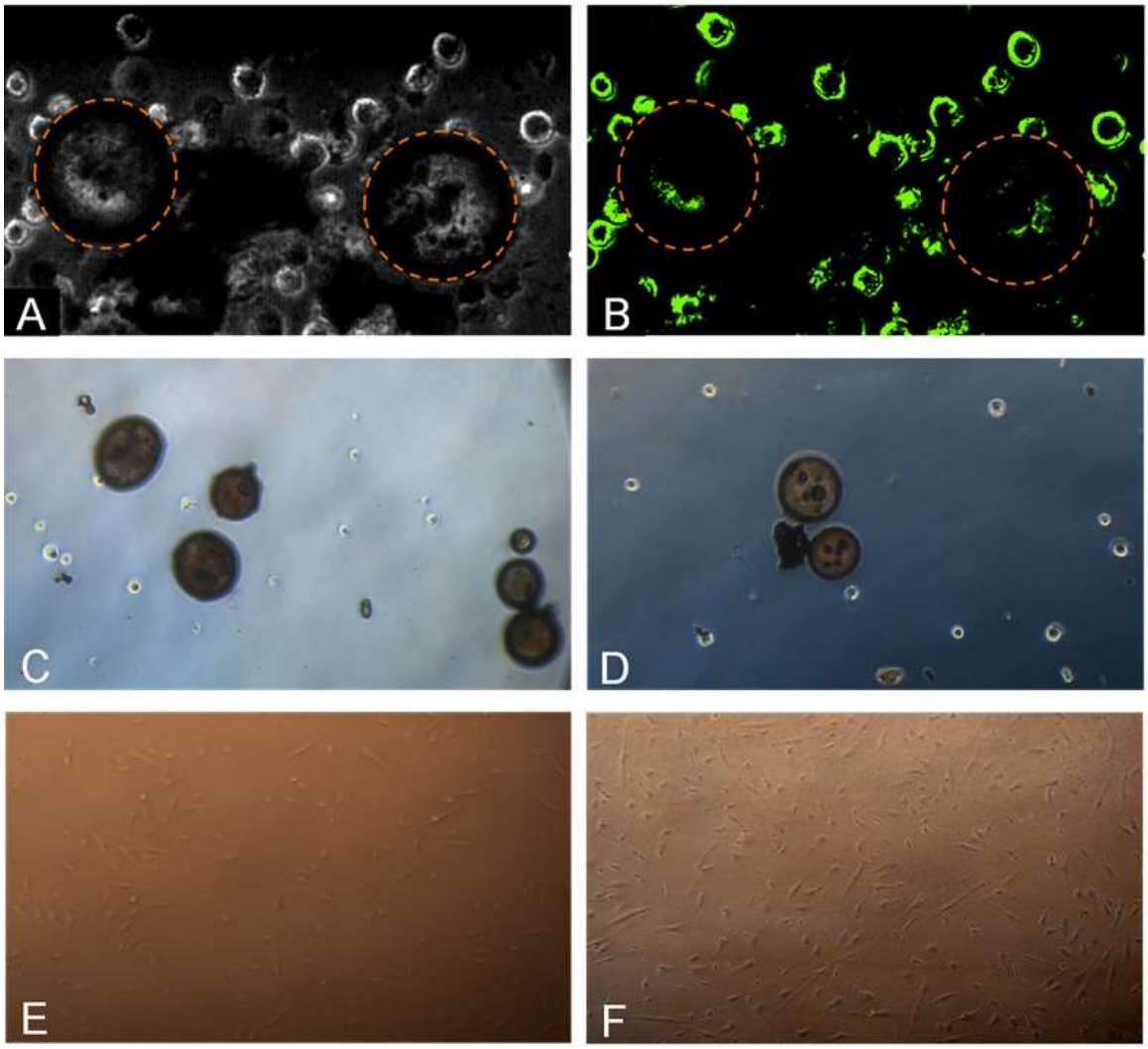

Figure 3.12 Cell detachment from MLMP surface. (A, B) Monochrome and LIVE/DEAD imaging, live cells stained in green with a small population on the surface of the particle (dashed lines representing MLMP outlines), (C, D) Microscopic images of detachment over time (30 and 60 minutes) and, (E, F) Growth and proliferation of detached cells at 7 and 21 days, respectively.

3.4 Isolation from Complex Cell Mixtures

EPC isolation from a complex mixture containing HDF cells and EPCs in a suspension was performed. Firstly, both the cell lines were tested for the presence of CD34 surface markers by immunostaining with fluorescently labeled CD34 antibodies. As observed in Figure 3.13, EPCs showed the presence of CD34 markers, while HDFs cells were not stained indicating the

absence of CD34 as a surface receptor. After the conformation of the presence/absence of CD34 marker on each cell type, the cells were combined in equal amounts to form a complex cell mixture, and cell isolation was performed using MLMPs. The isolated populations were then cultured and immunostained with Dil-Ac-LDL, FITC labeled CD34 and DAPI to analyze the specificity of EPC isolation with respect to CD34 conjugation over MLMPs.

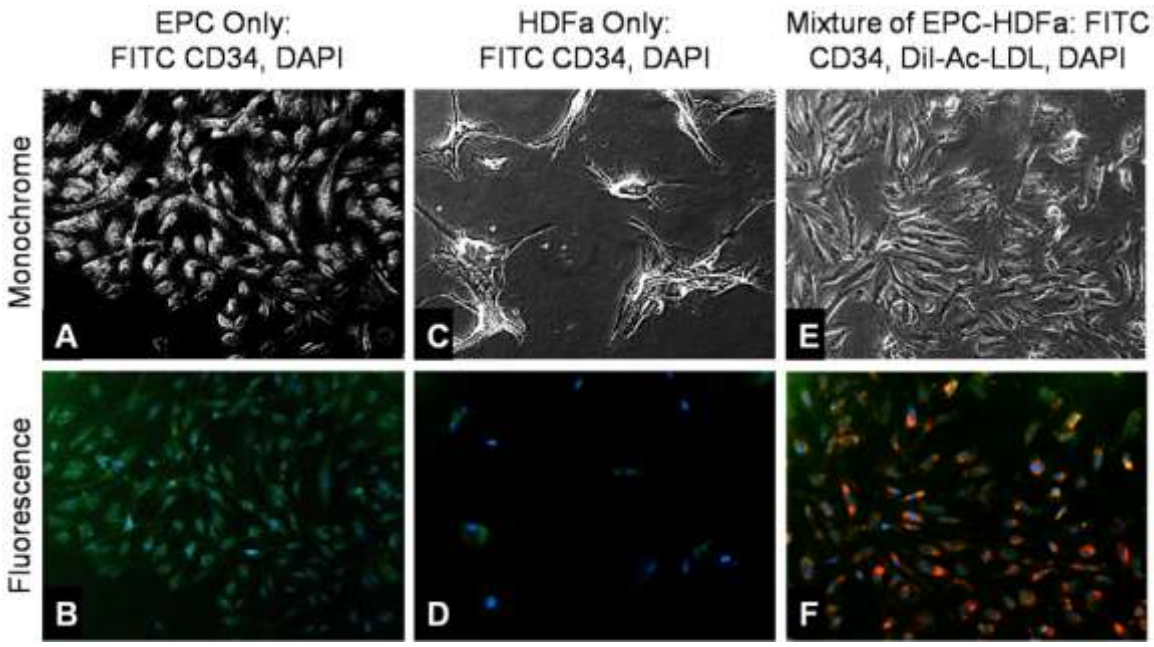

Figure 3.13 Immunostaining with FITC labeled CD34 and DAPI (A, B) EPC displaying bright green and blue fluorescence indicating the presence of CD34 surface marker, and (C, D) HDF cells stained with DAPI only and absence of CD34 markers. (E, F) Isolated cells showing the presence of CD34 marker by staining with FITC labeled CD34, DAPI as well as Dil-Ac-LDL.

Further, LIVE/DEAD assay and SEM images indicated the presence of cells on the particle surface as depicted in Figure 3.14. Images were taken at different stages of cell isolation; LIVE/DEAD staining indicated the increase in cell number over the particle surface with time. SEM image also verifies the presence of cells over the particle surface; extensions or sprouts [68] from the cells are observed and also the presence ECM being secreted by the cell perhaps due to minor stress exerted during rotation.

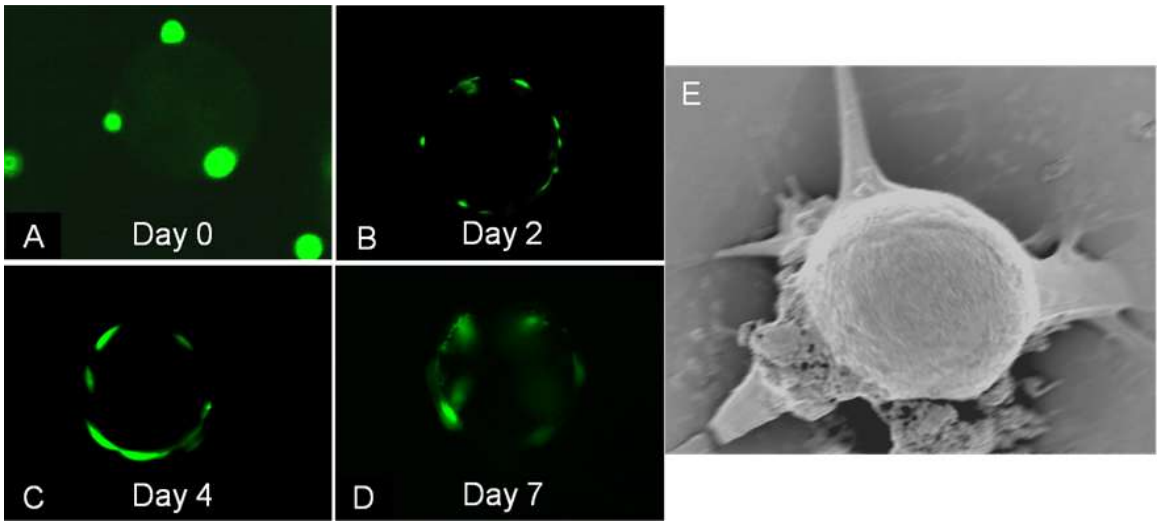

Figure 3.14 Cell-particle characterizations (A-D) LIVE/DEAD staining at different stages of proliferation and (E) SEM images of Cell-particle complex indicating attachment and proliferation of cells over the particle surface.

3.5 Blood studies: Cell isolation with Ficoll and MLMPs

So far only pictorial results were obtained after isolation of cells from a sample of blood. Figure 3.15 indicates the cells isolated from the blood by the use of MLMPs in comparison with that obtained using Ficoll. Large populations of red blood cells are observed within the culture during the first 2 days. At day 0, large populations of cells were obtained after isolation in both groups. These cells included some RBCs in addition to EPCs. After change of media at day 2, cell-particle complexes were imaged and slightly larger cells were observed growing on the particle, while few cells were seen in the culture obtained with the use of Ficoll due to the removal of RBCs and unattached cell populations. Further, at day 7 and day 14, cells were still observed growing over the surface, while compared to the Ficoll culture, the cells began to elongate with gradual proliferation. Extended quantitative analysis is part of our future studies.

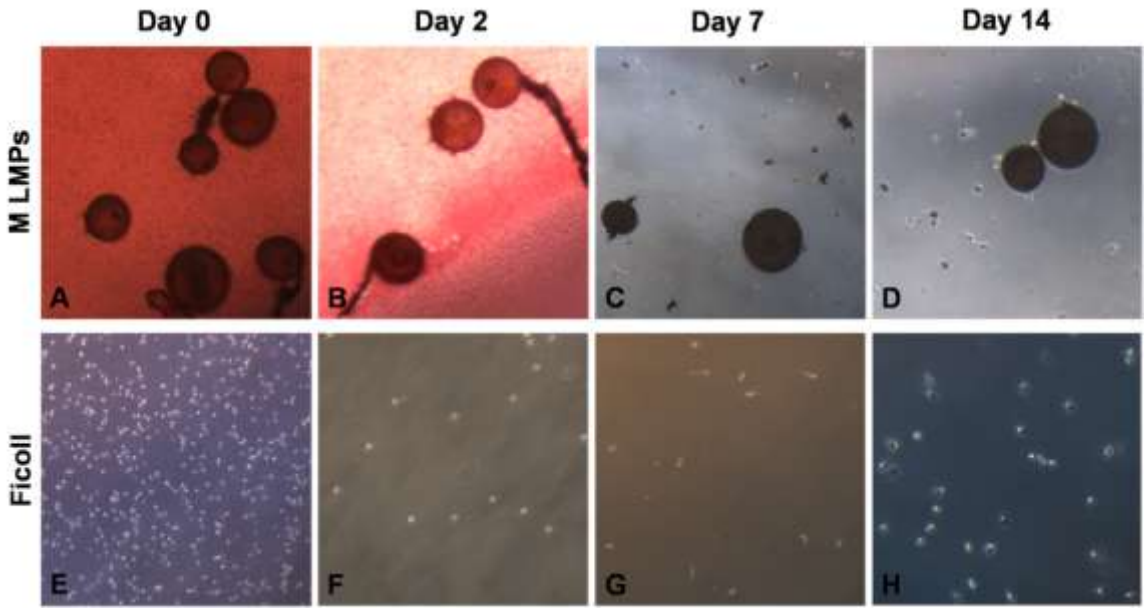

Figure 3.15 Micrographs of cells isolated from blood sample by MLMPs and Ficoll at 20X magnification: (A, E) Cells isolated at day 0 indicating large cell populations including RBCs, (B, F) at day 2 cells seen growing on the surface, (C, G) at day 7 cell culture, and (D, H) at day 14 cells seen growing distinctly on the particle surface.

3.6 Cell Expansion Study

Cell expansion using MLMPs was compared with the commercial cell expansion bead Cytodex3 using EPCs. Cell proliferation rates vary among the different groups as seen by the graph in Figure 3.16 (below). The graph was plotted by considering the initial seeding as 100%. MLMPs loaded with growth factors showed a significant increase in cell growth rates until day 4. The percent increase in DNA was approximately 390% at day 4, while cell proliferation until day 21 indicated a saturated profile. Further, cells cultured with MLMPs in media containing growth factors also showed similar profile, with lower values of 300 % at day 4 (p=0.0077). Comparing these results with cell growth over commercial Cytodex3 beads, a steady increase was observed up to 200% over time. Lastly, cells didn't grow when cultured with either MLMPs or Cytodex3 in media deprived of growth factors as growth curves indicated no increase in cell proliferation with 21 days.

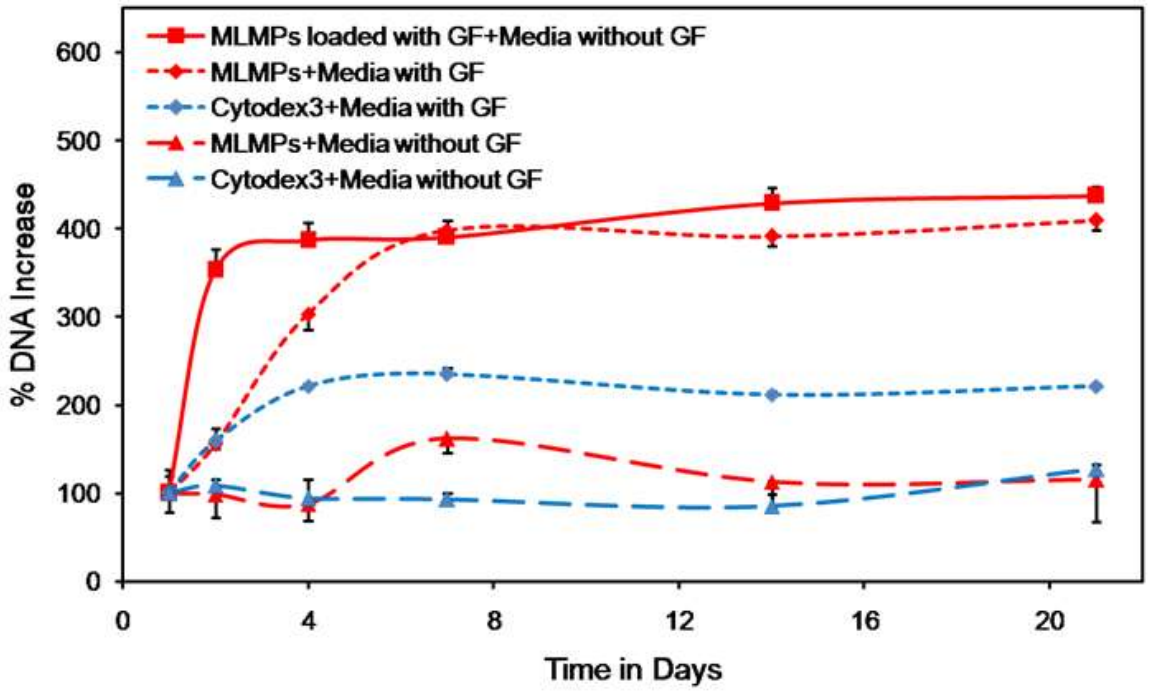

Figure 3.16 Cell expansion curves comparing proliferation rates over MLMPs and commercially available Cytodex3 microcarriers at different culture conditions.

CHAPTER 4

DISCUSSION

4.1 Polymer and Particle Characteristics

Culture environment of stem cells is very critical and plays an important role in cellular properties, expansion and differentiation [69-71]. The destiny of the stem cells cultured over the particles may be affected by many factors, thus the optimal culture conditions should be addressed. In order to achieve effective utilization of stem cells for regenerative medicine, interactions between the cell and the polymeric surface as well as microenvironments including physical and chemical topography have been shown to influence multiple cellular properties like cell migration, proliferation, lineage specificity, and cellular differentiation [70].

The temperature-sensitive polymer such as PNIPAAm have been well-studied for cell sheet engineering since PNIPAAm is known to possess reversible phase transitions with water, hydration or dehydration if the polymer chain takes place with changes in temperature [72, 73]. This polymer has been widely studied as particle coatings or as micelles for drug delivery applications [74, 75], and is used as an effective surface for cell growth and detachment [76]. The rationale of using PNIPAAm-AH as the outer most coating of the MLMP was taken from research done on cell sheet studies [77-79] and also from the fact that this polymer does not reveal an advert toxicity as seen in our study (Figure 3.1) and others [80-82].The LCST of the PNIPAAm-AH polymer is found to be 33ºC; this temperature is optimum for cell detachment from the particle surface by changing the temperature from incubation to room temperature. Moreover, temperature responsive cell detachment and mechanical dissociation is known to allow better subsequent adhesion over the surface in comparison to enzymatic digestion [83].

The LCST can be effected by the nature of substitute groups and their molar masses [84] as well as the length of the hydrophobic chain of the surfactant and its concentration [85].

As for the magnetic based multi layered particles, PLGA was used in the innermost core and loaded with the differentiation growth factor. PLGA was selected in our studies due to its well-known characteristics, including biocompatibility, biodegradation, and potential to provide a sustained release of a therapeutic reagent [55, 61]. The presence of magnetic particles facilitates the ease of separation of cell-particle complexes from the cell suspension. This magnetic property of MLMPs also possesses the advantage of ease in changing media and performing any type of experiments due to the fact that they can be immobilized over a surface in the presence of a magnetic field. This property is a major advantage over traditional microbeads like Cytodex3 microcarriers that require the use of centrifugation [52]. Cells seeded on the particle surface are protected from the magnetic nanoparticles (MNPs) due to the presence of the polymeric coating over the magnetic layer. This helps prevent the undesired ingestion of MNPs by stem cells. The polymeric layer does not compromise the magnetic property of the MLMPs as seen in the results obtained by VSM. Particle aggregation is critical with PNIPAAm particles in solution and primarily depends on the pH of the solution under consideration due to the presence of amine bonds and carboxylic groups [72]. This may account for the low number of cells isolated with increasing time as a result of reduced surface area.

As for the growth factors embedded in the shell and core, VEGF is known to have a proliferative effect on EPCs, while bFGF is the differentiation protein essential for effective maturation of endothelial stem cells into mature endothelial cells (ECs). The rationale behind loading VEGF in the shell was to increase the number of cells growing over the surface and then induce differentiation of these cells by bFGF release from the particle core. The release kinetics was as desired for effective proliferation and timely maturation as observed in Figure 3.9.

Further, conjugation with specific monoclonal antibodies was evaluated as seen in the fluorescence images of particles conjugated with labeled antibodies. In studies with isolation of EPCs, anti-CD34 was conjugated over the surface; according to several researches this is the most predominant and specific marker for human EPCs[19]. As a proof of concept, efficiency of MLMPs to isolate metastatic cancer cells like LAPC4 was also evaluated with CD117 conjugated MLMPs. Bioreactor rotatory tubes are used in our cell isolation and expansion studies instead of static cultures. Bioreactor rotatory tubes provide the advantage of uniform cell attachment over the 3D surface, compared to that observed under static seeding conditions, leading to higher seeding efficiencies and uniform cell attachment over the surface [86].

4.2 Cell Isolation and Detachment Optimization

Detailed optimization studies for cell isolation of EPCs were performed with respect to time, MLMP concentration and antibody concentration. These values were cross-referenced with a hemocytometer reading. Also, as an additional proof of concept, isolation was done with LAPC4 cells. Maximal cell isolation was obtained at 2 hours with 1 mg MLMPs and 3 µg/ml antibody concentration (Figure 3.10). Isolation optimization was essential for efficient and more accurate analysis for further cell studies like that of isolation from complex cell suspensions. Decreased isolation efficiency was seen during later time points at 4 and 6 hours; this may be due to the particle aggregation. Loss of antibodies from the surface also resulted in loss of the cell-antibody complex, reducing the final cell count. Further, as the results indicated, 80% of the cells detached initially from the surface of the particle within the first 30 minutes. The same results were observed with research on cell sheet engineering over the polymer surfaces under static conditions[60]. This is primarily due to the hydration of the polymeric surface.100 % detachment efficiency was not obtained; this may be accounted for absence of temperature-sensitive polymer coating in some particle surface areas, causing the cells to attach over the PLGA layer. The cells attached over the PLGA surface do not detach by change in temperature,

and proteolytic enzymes may be necessary. Images obtained by LIVE/DEAD staining indicated substantial cell populations over the particle surface, but these cells appear rounded. This may be a result of cell detachment from the surface due to the lowering of surrounding temperature. This limitation during imaging was tried to be kept at minimum by controlling environmental parameters. Isolation form complex cell mixtures were analyzed using immunostaining, and expected results on EPC isolated populations were also obtained.

4.3 Blood studies: Cell isolation with Ficoll and MLMPs

Studies on blood were performed in focus of isolating EPCs, and isolated cells were viewed only by microscopic observation, as part of our preliminary *in vitro* study. Cells were seen growing over the particle surface; however, these cells must be further analyzed for surface markers specific to EPCs and endothelial cells as well as their physiological functions. Further studies on isolated cells will include immunofluorescence antigen staining and quantification by FACS analysis.

4.4 Cell Expansion Study

Studies were performed on 5 different groups at varying culture parameters; this was done to observe if there are any differences with cell growth when growth factors supplied in the media with that loaded within the particle. Groups with growth factors within the particle or media were also compared against those deprived of growth factor entirely. The commercial Cytodex3 beads were used for comparison with our MLMPs. The results indicate that MLMPs supplied with growth factors display a significant level of compatibility towards stem cell growth. Specifically localized supply of growth factors to the cells growing over the particle seems to have a better effect on cell proliferation thus emphasizing the benefit of growth factor loading within the particle compared to that supplied in the media. This is not only advantageous for cell

proliferation but also in minimizing the use of these expensive proteins by adding it to the media.

As for cellular differentiation, studies indicated that direct cell-cell contact has an effect on stem cell differentiation [87], while cellular gap junctions are known to play an important role in cell-cell communications and differentiation. The surface properties of the biomaterial over which stem cells are cultured and grown is known to influence differentiation even in the absence of differentiation molecules like growth factors. Biomaterial properties include degradation kinetics, molecular compatibility and porosity [88]. The differentiation of cells over the MLMP surface must therefore further be analyzed with respect to time and the effect of growth factors.

CHAPTER 5

SUMMARY, LIMITATIONS AND FUTURE WORK

5.1 Summary highlighting the advantages

Our results demonstrate that these magnetic based microparticles have the capacity and efficacy to isolate a required and specific stem cell lineage. MLMPs have shown several advantages over traditional isolation and expansion techniques. The particles support substantial cell adhesion, growth, and expansion over a period of time. Improved cell attachment over the particle surface has been proved by different imaging techniques. Cells were imaged on the polymeric surfaces by immunofluorescence, H&E staining, and SEM imaging. LIVE/DEAD images indicated a substantially high population of live cells attached over the surface, but no dead cell population was observed. SEM images also indicated cells grown over the surface, but ECM production and sprouts from the cells were observed perhaps due to the minor stress exerted as a result of rotation. Growth factor release characteristics observed seems beneficial in cell expansion and proliferation over time as demonstrated by the results from the elaborate expansion study. Lastly, the cells cultured after isolation showed no differences in morphology and were capable of proliferating under *in vitro* conditions.

5.2 Limitations and Alternatives

Although MLMPs facilitate isolation, adhesion, and proliferation of the stem cells over its surface based on their unique advantages, there are some limitations that may be encountered with this system.

- Synthesis of MLMPs involves the use of organic solvents and also elaborate fabrication steps, this intense procedure may compromise the bioactivity of the growth factors embedded in the core as well as the shell [88]. The bioactivity of these growth factors must be analyzed.
- One of the major concerns was the effects of the degradation products of the particle components on the stem cells, PLGA undergoes hydrolytic degradation resulting in production of its acidic monomers [89]. This acidity on the cell culture environment may be counteracted by suitably adjusting the pH of the media used.
- Another limitation is the development of cell to cell junctions forming cell sheet contiguity over the particle surface[79], leading to inefficient cell detachment. These junctions created may be broken apart by the use of some digestive enzymes if needed.
- Detachment of cells from the particle may be inefficient due to attachment to the antibody. Cells attached to antibodies may be detached based on the principle of competitive binding, by the use of synthetic peptides.
- Particle aggregation may be a limitation for EPCs growing on the particle surface. This can be overcome by culturing cell-particle complexes in spinner flasks and other bioreactors to keep particles dispersed in suspension and apart from each other.
- Particles need to be further analyzed for successful uniform of polymer coating over the entire surface of the microcarrier, as an uneven coating may be accounted for low cell isolation and detachment efficiencies of these particles.

5.3 Future Prospects

Summarizing the advantages and limitations of our novel isolation system has helped draw an outline towards future studies and the scope of the project. Since the cell expansion study has been successfully performed for our microparticles, the next step would be to quantify the cell differentiation over a time course. As previously described, the topography of the

polymeric surface can also have an effect on stem cell differentiation[88], thus a prospective study is to analyze the stage at which differentiation begins and whether the occurrence of any undesired differentiation takes place. The cell differentiation characteristics will be studied in detail with FACS array analysis and immunostaining for specific expression of surface markers and antigens. SEM images will also be taken from time to time to observe the change in particle morphology and its interaction with the cells on the surface.

Coating of the particles with fibronectin or matrigel should be studied to enhance the cell adhesion over the particle surface. This may be beneficial for enhancing isolation of stem cells. Matrigel is considered to have excellent properties with respect to cell surface addition [90].In order to improve the cell adhesive properties over the PNIPAAm surface, treatment with FBS or other cell adhesion proteins is helpful in improving adhesion of the cells with the culture surface. Lastly, efficient and improved stem cell isolation may be achieved perhaps by the presence of a combination of antibodies[91, 92]. A study on cell isolation with MLMPs conjugated with two of the most closely associated antibodies, for example, CD34 and AC133 for EPC, must also be performed. The use of these microcarriers cultured with stem cells may also extend opportunities in 3D scaffolding and other various regenerative fields, and *in vivo* stem cell delivery.

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BIOGRAPHICAL INFORMATION

Sonia Santimano was born in Doha, Qatar in May 1989. She graduated with a Bachelor's degree in Biomedical Engineering from K.L.E.S. Engineering College, Visveswaraya Technological University, India in June 2010. To accomplish her goal aimed at a future in medical based research and clinical frontier, she joined the University of Texas at Arlington in Fall 2010, focused on achieving a graduate degree in Biomedical Engineering. She began her research study in the Nanomedicine and Drug Delivery Laboratory of Dr. Kytai T. Nguyen, whose expertise is in the field of nanotechnology, drug delivery and tissue engineering. She worked on several projects encompassing those related with cancer diagnosis and therapy as well as stem cell based techniques. Her research interests include drug delivery, stem cell techniques, biomaterials and nanotechnology. Her future plan is to continue a career in research encompassing drug delivery systems and stem cell technology.