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Novel method for delivery of Stem cell-derived growth factors for Heart therapy applications: *In vitro* and *In vivo* analysis

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Abstract

Cardiovascular diseases still remain the leading cause of morbidity in Canada with heart failure and stroke being two of the three main causes of death. Although, medical and surgical options for treatment and care after the onset of the disease do exist, more than half the patients suffering from heart disease die within 5years of diagnosis. Currently, heart transplant still remains the last resort for patients with end-stage heart failure, with the demand for organ donors alarmingly exceeding the supply. Numerous groups have now turned towards investigating the possibility of stimulating the regenerative ability of the heart through various strategies, with cellular therapy and specifically stem cell regenerative therapy increasingly gaining popularity. Several clinical studies demonstrate the possibility of injecting adult stem cells to induce therapeutic angiogenesis and revascularization and in turn promote damaged tissue regeneration. Although these trials show that stem cells possess the potential to facilitate tissue repair, there has been uncertainty over the mechanisms responsible for this regenerative effect produced by the transplanted stem cells. Furthermore, recent evidence has emerged suggesting that a paracrine effect, created by growth factors secreted by the injected stem cells, is actually the key mediator in assisting therapeutic regeneration, which has now been demonstrated both *in vitro* and in small/large animal models through direct injection of the stem cell harnessed factors. However, other groups have also observed that bolus injection of individual or dual proteins, although showing initial success, does not have a prolonged effect enough to promote complete tissue regeneration. Thus, a novel method of delivering the stem cell harnessed factor cocktail would be needed to augment the effect of the stem cell derived growth factors delivered at the target site, which forms the main goal of this thesis. Specifically, we investigate the strategy of using controlled delivery particle systems which not only provide a continuous release but also protect the encapsulated factors from the harsh *in vivo* conditions. The use of our stem cell harnessed factor cocktail loaded polymeric system, mimicking the paracrine effect created by injected cells, could completely replace the concept of stem cell administration and pave the way for a new strategy in cellular therapy.

Résumé

Les maladies cardiovasculaires sont la première cause de morbidité au Canada. Les insuffisances cardiaques et les accidents cardiovasculaires représentent les majeures causes de mortalité. A ce jour, la transplantation cardiaque demeure l'ultime recours aux patients en phase terminale, malgré la demande alarmante de donneurs organes excédant l'offre. De nombreuses stratégies sont actuellement étudiées. La thérapie cellulaire, notamment la thérapie régénérative de cellules souches, est une stratégie prometteuse en plein essor. Plusieurs essais cliniques ont démontré que l'injection de cellules souches adules peut induire l'angiogenèse et la revascularisation des tissus. Cela permet de promouvoir la régénération des tissus endommagés. Malgré l'efficacité démontrée de ces études, les mécanismes d'action demeurent incertains. De récentes études *in vitro* et *in vivo* ont suggérés que les facteurs de croissance secrétés par les cellules souches injectées sont à l'origine d'un effet paracrine clé pour la régénération thérapeutique. D'autres groupes ont observés que l'injection d'un bolus de protéines peut initier la régénération thérapeutique. Cependant, cet effet n'est pas assez maintenu pour promouvoir la régénération complète des tissus. Développer un nouveau système de délivrance contrôlé pour administrer le cocktail de facteurs de croissance produits par les cellules souches constitue une nouvelle approche prometteuse pour augmenter la régénération thérapeutique. Ceci constitue l'objectif principal de cette thèse. Nous effectuons des recherches sur la stratégie d'utilisation de systèmes de délivrance contrôlés pour administrer les facteurs de croissance dérivés de cellules souches. Ceci a pour but de simuler et augmenter l'effet paracrine induit par l'injection de cellules souches. L'encapsulation de ces facteurs dans une matrice polymérique permet de les protéger des conditions sévères rencontrées *in vivo* tout en les libérant en continue dans les tissus ciblés. Ce cocktail de facteurs de cellules souches encapsulés pourrait complètement révolutionner le concept des cellules souches. Ces recherches ouvrent la voie à une nouvelle stratégie de thérapie cellulaire.

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Preface

In accordance with the McGill University Thesis Preparation and Submission Guidelines, I have taken the option of writing the experimental section as a compilation of original papers either published or suitable for publication.

In this thesis, manuscripts of original papers are presented in chapters 4, 5 and 6 and are subdivided into sections including abstract, introduction, materials and methods, results, discussion and conclusion. In addition, a common abstract, general introduction, literature review, general discussion, summary of observations, overall conclusions, recommendations and future applications are included in the thesis as required by the guidelines.

List of Abbreviations

Ang-1	Angiopoietin-1
AFM	Atomic Force Microscopy
BMSCs	Bone marrow derived stem cells
BSA	Bovine serum albumin
CM	Conditioned medium
DCM	Dichloromethane
DMEM	Dulbecco's Modified Eagle Medium
ECM	Endothelial cell medium
FDA	Food and Drug Administration
FBS	Fetal bovine albumin
GFs	Growth factors
HSA	Human serum albumin
HGF	Hepatic Growth Factor
HUVECs	Human umbilical vein endothelial cells
hASC	Human adipose stem cell
hBMSCs	Human bone marrow derived stem cells
hVEGF	Human vascular endothelial growth factor
hbFGF	Human basic fibroblast growth factor
MI	Myocardial infarct
MSCs	Mesenchymal stem cells
MSCM	Mesenchymal Stem Cell Medium
MPs	Microparticles
MP/coll	Microparticles embedded in collagen matrix
NPs	Nanoparticles
o/w	Oil-in-water
PLGA	Poly-(lactic-co-glycolic acid)
PVA	Poly-(vinyl alcohol)
PDI	Polydispersity index
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy

w/o/w Water-in-oil-in-water

Units:

ng nanogram

µg microgram

nm nanometer

µm micrometer

mL milliliters

1 General Introduction

1.1 Overview

Cardiovascular diseases account for almost 30% of deaths in Canada with heart failure and stroke being two of the three leading causes of death[1]. Although great strives have been made to provide improved acute medical care and primary and secondary prevention after the onset of cardiac disease, heart failure due to heart muscle cell or cardiomyocyte destruction still remains the first cause of death in several developed countries. As a result, the need for organ donors is on the rise, with heart transplantion still being the only resort for end-stage heart failure patients. However, with demand alarmingly exceeding the availability of organs and the experimental phase after xenotransplanation, quite often even this strategy does not fulfill the requirement of the patient [2]. To overcome these problems, numerous studies demonstrating the ability of the adult human heart to naturally generate and repair myocytes in the damaged heart have arisen [3] with investigations in alternative therapeutic approaches, such as protein therapy, gene therapy and stem cell therapy as possible strategies to target stimulation of this process[4]. These approaches aim to promote tissue neovascularization and in turn organ regeneration and remodeling after disease.

Cellular therapy has especially gained recognition with numerous groups demonstrating the possibility of injecting mesenchymal stem cells (MSCs) to promote the repair and regeneration of damaged tissue by inducing therapeutic angiogenesis and revascularization [5, 6]. The mechanisms by which these injected stem cells function includes differentiation, cell fusion events and paracrine effects through the secretion of cytokines or growth factors (GFs) [7].However, there remains controversy over the mechanisms underlying these regenerative effects created by the stem cells with the rise of evidence supporting the paracrine effect, involving the secretion of biologically active factors from the administered cells, as the key mediator in promoting tissue healing and repair following myocardial infarct [8, 9]. Several groups have obtained significant results supporting the paracrine hypothesis, demonstrating its potential in stimulating myocardial remodelling via stimulation of angiogenesis, suppression of cardiomyocyte apoptosis with potential application in tissue regeneration therapy [8, 10-13].

It is hypothesized that the use of this stem cell derived growth factor cocktail or conditioned medium (CM) could evade the concept of stem cell administration for tissue regenerative purposes and subsequently the related drawbacks to their use such as low cell viability after injection, local immune responses and the increased release of inflammatory mediators in turn disrupting homeostasis within tissue [14]. Furthermore, studies also highlighted additional drawbacks such as the ability of these stem cells to suppress immune responses and in turn promote tumor formation and spread [15]. However, other groups also demonstrate that, although initial results with pro-angiogenic proteins directly injected as a bolus have been promising, the effect is short lived due to protein instability or denaturation after administration [16, 17]. Moreover, these proteins have to injected multiple times to achieve modest success.

Thus, the need for an efficient delivery mechanism, aimed at enhancing the therapeutic effectiveness of the administered stem cell CM becomes imperative. It is essential that an appropriate delivery vehicle is used which not only provides a sustained and controlled release of the stem cell-derived growth factors from at the site. Although several strategies have been applied for the development of appropriate vehicles for efficient and sustained protein delivery such as the use of polymeric scaffolds, the use of controlled drug delivery polymer systems such as microparticles (MPs) and nanoparticles (NPs) for potential application in promoting tissue regeneration has gained significant interest[18]. Such polymeric particle systems not only allow controlled release of the encapsulated factors but also protect the proteins from denaturation in the harsh *in vivo* conditions.

The main goal of this thesis is to investigate the novel strategy of using biocompatible controlled delivery polymeric nano/microparticles to enable a sustained release of stem cell harnessed growth factors for potential application in myocardial tissue repair and remodeling therapy. Such a strategy of using controlled release particles would allow a slow continuous release and also protect the encapsulated factor cocktail from the harsh *in vivo* environment for a more prolonged therapeutic effect at the damaged tissue site. This technique could completely replace the stem cell administration and the drawbacks related to their use, such as poor retention in the myocardium and generation of local immune response, by directly mimicking the paracrine effect created by the injected cells.

1.2 Thesis research Objectives

The primary objective of thesis is to investigate the novel strategy of using polymeric controlled release particles for delivery of growth factors harnessed from stem cells with potential heart therapy applications. The specific research objectives are:

a) To examine the effects of delivering a dual combination of growth factors using a polymeric particle system
b) To design, develop and characterize a suitable stem cell derived multiple growth factor loaded polymeric particle system for cardiac tissue re-modeling
c) To investigate the potential of the developed particle system to promote *in vivo* myocardial angiogenesis
d) To develop and access the benefit of embedding the developed growth factor loaded particle system into a polymeric hydrogel

1.3 Outline of thesis

This thesis is divided into 7 chapters. Chapter 1 provides the general introduction and research objectives of this thesis, followed by an extensive literature review of the subject given in chapter 2. Chapter 3 provides the preface for Chapters 4, 5 and 6. Chapters 4-6 are original research either published or to be submitted for publication. The main investigations and observations performed to achieve the research objectives are included in the original papers. Chapter 7 provides a general discussion. The findings of the thesis studies have been summarized in Chapter 8 followed by the conclusions based on the thesis work. Recommendations and future applications have been included in Chapter 10.

2 Literature Review

2.1 Regenerative medicine

Regenerative medicine is defined as the process of replacing or regenerating damaged tissues or organs to restore or establish normal function. The main goal of this field is to promote the healing and regeneration of tissues and organs through the stimulation of the individual's own repair mechanisms at the damaged site[19]. It is aimed that the principles of regenerative medicine could one day provide the solution to problems such as shortage of donor organ availability, where demand alarmingly exceeds availability, as well organ transplant rejection.

2.2 Cardiovascular diseases

In 2006, cardiovascular disease accounted for 30% of all deaths in Canada (69,019 deaths – or more than 69,000) and still remains the leading cause of deaths with 30% of all male deaths and 31% of all female deaths. Statistics show that men tend to suffer from cardiac disease early in life as compared to women who are more likely to develop the disease after menopause. Of all cardiovascular deaths in 2006, 54% were due to ischemic heart disease, 20% were because of stroke and 23% were due to heart attack (Statistics are based on 2006 data, the latest year available from Statistics Canada). Heart failure in a patient is the result of gradual death of heart muscle cells or cardiomyocytes after the onset of a heart attack or cardiac hypertension. Certain drug treatment options do exist for patients after a heart attack or heart disease to prevent or dissolve blood clots, lower blood pressure, prevent and treat angina symptoms and improve cardiac contractions. Surgical procedures also enable diagnosis and treatment of heart disease such as coronary angiography and angioplasty, bypass surgery, coronary stenting, heart valve defect correction surgery, implanting of cardiac defibrillators and heart transplants. However, even with the advances made in surgical procedures, drug treatment, mechanical assistance devices, statistics show that more than half of the congestive heart failure patients die within five years of the first diagnosis. Heart transplant still remains the only resort for end-stage heart failure patients and the need for organ donors is on the rise with the requirement severely exceeding the supply[2]. With goals to provide a solution to this huge problem, numerous approaches are being investigated to promote stimulation of the regenerative capacity of the myocardium due to the presence of cardiac progenitors or stem cells such as protein therapy, gene therapy and stem cell therapy to promote tissue neovascularization and in turn organ

regeneration and remodeling after disease[20-22]. Although several research groups have provided evidence of the potential of these strategies, these studies are still insufficient and more research is necessary to develop methods that could avert the onset of cardiac failure after MI[4].

2.3 Neovascularization

The formation of new blood vessels plays a key role at the time of embryo development and the physiological repair of damaged tissue [23]. New vessel development is normal and essentially observed during wound healing after trauma and ischemic tissue restoration and repair. It is therefore imperative that stimulation of new vessel growth is site specific and organ specific as the formation of abnormal blood vessels contributes to a number of diseases [24, 25]. Three major processes that contribute to the growth of new blood vessels or neovascularization are vasculogenesis, angiogenesis and arteriogenesis [26]:

2.3.1 Vasculogenesis

Initially, vasculogenesis was thought to occur at the time of embryogenesis where a differentiation of embryonic mesenchymal cells, for instance endothelial precursor cells or angioblasts, into endothelial cells occurs as well as at the time of de novo development of blood vessels. However, now it is well known that this process contributes to adult neovascularization as well induced by VEGF and other angiogenic factors. The new blood vessels so formed are composed mainly of endothelial cells and are referred to as the capillary plexus.

2.3.2 Angiogenesis

It is one of the most important natural processes in the body used for wound healing, tissue repair and development. The process involves formation or growth of new blood vessels by sprouting from pre existing small vessels in adult and embryonic tissue or by an intravascular subdivision process called intussusceptions [27]. When local angiogenic stimuli increase, the activation of the endothelial cells found in pre-existing vessels occurs with subsequent vasodilatation. This is followed by the disruption of the basement membrane of the pre-existing capillaries encompassing endothelial cells. The cytoplasmic process extends from the activated endothelial cells, directing their migration and sprouting to the extra vascular space moving towards the angiogenic stimuli [28]. The cells then elongate and align to form capillary sprouts and then form a lumen finally connecting with the neighboring vessels. The presence of angiogenic growth factors or proteins plays a key role in promotion of angiogenesis during tissue repair and

recovery. Angiogenic growth factors are called so because of their ability to induce the proliferation of various cells *in vitro*, which contribute to the process of angiogenesis *in vivo* as shown by studies of animal models. Some of the characteristics of key pro-angiogenic proteins VEGF, basic fibroblast growth factor (bFGF) and Angiopoieins are given below:

2.3.2.1 *Vascular endothelial growth factor (VEGF)*

The VEGF family members are a family of homodimeric glycoproteins structurally related to the platelet-derived growth factors (PDGF), vital for the normal regulation of vasculogenesis, angiogenesis and blood vessel maintenance[29]. These proteins are essential both at the embryonic vasculature development stage, where the VEGF family members and their receptors are secreted by the surrounding cells and mature tissues during new blood capillary formation in adults[30]. The most important isoform of this family VEGF-A (mostly called VEGF), first identified by Ferrara et al., plays a key role during early blood vessel formation and angiogenesis[31]. VEGF acts through interaction with endothelial-cell-specific tyrokine kinase receptors, VEGFR-1 and VEGFR-2 selectively which have been shown to be overexpressed in pathological angiogenesis [32]. However, several studies show that it this angiogenic effect involving endothelial cell mitogenesis, migration and survival is mediated by certain signaling pathways generated through the specific binding of VEGF to VEGFR-2[33]. Extensive *in vivo* studies have also shown the ability of VEGF to stimulate endothelial cell proliferation and promote cell survival[29].

2.3.2.2 *Basic fibroblast growth factor (bFGF)*

The fibroblast growth factor (FGF) family, produced by endothelial cells and smooth muscle cells, consists of an increasing number of peptide growth factors with several biological effects and cellular targets [34]. Two family members which are the most commonly researched isoforms, acidic fibroblast growth factor (FGF-1) and FGF-2, have a strong affinity for heparin-like glycosaminoglycan of the extra cellular matrix and have been studied in-depth for their effects on vascular cells [35, 36]. Extensive evidence indicates that both FGF-1 and FGF-2 are potent angiogenic factors, promoting *in vitro* proliferation and differentiation of endothelial cells, smooth muscle cells and fibroblasts in addition to providing stimuli for therapeutic angiogenesis *in vivo*.

2.3.2.3 *Angiopoietin*

Angiopoietin 1 (Ang-1) was first isolated by Davis et. al. using the novel secretion-trap cloning approach in 1996 and has since then been the focus of extensive studies dedicated towards the microenvironments involving ischemia and angiogenesis [37]. This angiogenic factor, secreted primarily by smooth muscle cells close to the blood vessels, is also generated by astrocytes and pericytes [38]. The key function of this protein involves promotion of vessel maturation of newly formed blood vessels through branching during the post-sprouting phase of vessel development in addition to stabilization of existing vasculature. The Ang-1 ligand has a strong affinity towards the tyrosine kinase receptor Tie 2 present on endothelial cell surface[39]. Widely expressed in the embryonic and adult endothelium these Tie 2 receptors phosphorylate at the time of vascular remodeling such as during wound healing and ovulation as well in endothelial cells[40]. These receptors are known to play an essential role in endothelial cell stabilization and thus in turn significantly affect angiogenesis. Importance of the function of Angiopoietins in cardiovascular disease research is on the rise. The level of Ang-1 in the heart increases as it moves from extensive neonatal to limited adult remodeling [41].

2.3.3 Introduction to therapeutic angiogenesis, the currently used methods to enhance angiogenesis: potentials and limitations

The clinical use of methods to enhance or promote the development of collateral blood vessels in ischemic tissue termed as "Therapeutic Angiogenesis" has been the focus of study for several groups, with intense research being carried out on improvement strategies [26, 42]. The main aim to this angiogenic treatment is to provide an alternative to coronary artery bypass surgery or percutaneous coronary interventions which pose a high risk post surgery and it is hypothesized that this technique, if used in combination with surgery, could promote neovascularization and healing especially in patients suffering from ischemia.

The delivery method is essential for the efficient and effective therapeutic angiogenesis to occur with the following characteristics [43] :

- The therapeutic agent should have no chronic side effects and be targeted specifically to the ischemic tissue with high local concentration.
- It is imperative that the tissue is also exposed for a sufficient amount of time to the agent.

- Re-administration of the pro-angiogenic agent should be feasible and non invasive. For instance, studies have shown that during development the secretion of VEGF-A is very accurately regulated and even slight changes in the protein levels lead to abnormalities during development and embryonic death [44, 45].

Thus three major ways that are extensively being researched to apply pro-aniogenic agents and in turn promote therapeutic angiogenic therapy are protein therapy, gene therapy and cellular therapy [26, 42].

2.3.3.1 *Protein based therapy*

Protein based therapy involves the administration of pro-angiogenic growth factors or proteins such as VEGF and bFGF at the site of injury[46]. Currently the administration of proteins provides several advantages such as:

- Ease of protein dosage modulation in most clinical settings compared to gene therapy, which is hindered by the lack of a regulable expression vector.
- No exposure to genetic material
- Ease of re-injection of the protein agent
- Decreased risk of the immune response

Although protein therapy has many advantages, there are still many technical problems associated with protein administration, including short half life, deactivation on exposure to the harsh environmental conditions *in vivo* and optimization of purification which have to be overcome if this technique is to gain success.

2.3.3.2 *Gene based therapy*

Gene therapy through the use of viral vectors (for gene delivery) has been used to manipulate gene expression and target specific diseases. This strategy provides an attractive alternative to the use of proteins because it enables a prolonged and augmented exposure of growth factors at the target site locally after just a single administration. In one study, the injection of phVEGF165 gene into the stunned heart of a porcine model of ischemic heart led to an improved collateral blood flow [47]. In another study, the administration of adenovirus encoded with VEGF121 gene again into a stunned heart model led to an improved cardiac function with increased blood flow [48].

However, even this therapy has several disadvantages to its use especially with potential concerns of immune and inflammatory responses to viral vectors which has been shown in several studies [26]. For example in one study, aimed at treating brain tumors active brain inflammation as well as persistent transgene expression was observed after the use of adenovirus as a viral vector[49]. Another major disadvantage to the use of gene therapy is the lack of control over gene expression [50].

2.3.3.3 *Stem cell therapy in therapeutic angiogenesis: potentials and limitations*

The main objective of stem cell therapy is to promote healing of the damaged organ tissue and in turn restore functionality via direct transplantation of adult stem cells at the target site[51]. This technique involves the administration of cells to heal and repair the damaged ischemic tissue, with the main hypothesis being the ability of stem cells to re-new and differentiate into other cell types of the surrounding environment. There is extensive ongoing research testing the capacity of stem cells to promote regeneration and replace diseased and damaged tissue both through *in vitro* and *in vivo* animal models. Several studies using bone marrow derived stem cells (BMSCs) have shown to significantly improve organ function after disease or trauma through mechanisms including cell fusion, differentiation and paracrine effects [52]. Administration of the BMSCs into the injured organ led to augmented novascularization, anti- inflammatory and anti-apoptotic effects [53, 54]. However, the mechanisms behind this stem cell regenerative nature after administration is still unclear with several hypothesis such as lineage specific differentiation, transdifferentiation into cardiomyocytes and the secretion of growth factors or paracrine effects [53, 55].

Currently, the hypothesis that paracrine mediators, i.e. growth factors secreted by the implanted stem cells, contribute to myocardial remodelling via stimulation of angiogenesis and suppression of cardiomyocyte apoptosis is gaining attention and popularity with evidence from numerous groups [8, 10-13]. For instance, after the injection of stem cells, newly developed cardiomyocytes and vascular cells were formed and improved heart function is observed so soon that it could not be credited to cardiac tissue regeneration i.e. cellular fusion and diffusion events are observed to be very irregular and indirect paracrine effects are proposed to be the cause. Furthermore, studies have also shown that under serum and oxygen starvation culture conditions, a wide variety of biologically active pro-angiogenic growth factors or cytokines are secreted by MSCs and found in the MSC-conditioned culture medium (MSC-CM) [56] capable of promoting

tissue regeneration and healing. This stem cell generated growth factor cocktail has also been shown to support *in vitro* growth and migration of endothelial cells and vascular smooth muscle cells.

2.3.4 Arteriogenesis

It is defined as a rapid proliferation of pre-existing collateral vessels and these vessels have fully developed tunica media [27]. It is a mature type of neovascularization which can restore perfusion to an ischemic area. Several studies have shown that for arteriogenesis i.e. for stable and mature blood vessel formation, combination of the activity of numerous growth factors is needed (**Figure 2-1**) [42].

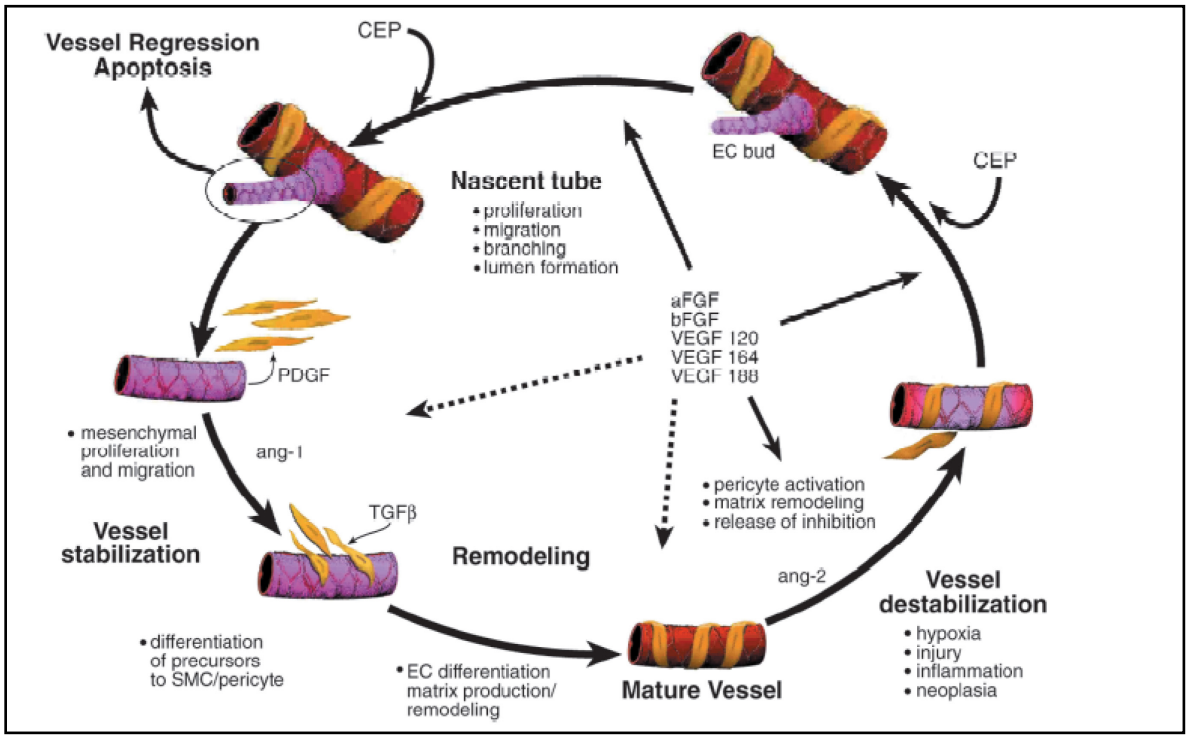

Figure 2-1: Schematic illustrating the factors which promote the formation of a stable blood vessel.

2.4 Introduction to biodegradable and biocompatible polymeric membrane delivery particles and their potential in controlled protein release

2.4.1 Rational for use of polymeric controlled drug delivery particles

Numerous studies have shown that the administration of pro-angiogenic cytokines or growth factors such as VEGF, FGF or HGF (Hepatic Growth Factor) [57], under an appropriate dosage can up-regulate angiogenesis by signaling endothelial cells to undergo proliferation, migration,

and differentiation to form new blood vessels and in turn promote tissue revascularization and repair. These angiogenesis promoting growth factors have been administered either as natural recombinant human proteins or by gene transfer [58, 59]. In several clinical studies it has been demonstrated that the VEGF protein when injected as a bolus showed some disappointing clinical results [17, 60-62] even though pre-clinical animal models and initial clinical trials have suggested a beneficial effect [16, 63]. Data demonstrated a short-lived effect with high instability (such as oxidation, formation in a physiological environment) of the protein. Also as the therapeutic effects of the protein can only be achieved at extremely high doses, through multiple administrations, and it in turn results in side effects such as hypotension, retinopathy, or progression of malignant tumors [60, 64]. Moreover, using the gene delivery strategy through naked plasmids or integrative viral vectors leads to low efficacy and concerns such as the risk of genome integration, limiting application of this method in a clinical setting [60, 61, 65].

The use of an alternative approach is thus imperative, which would allow sustained and controlled release of growth factors, with protein concentration maintained within the therapeutic window so as to prevent some of the problems described, and at the same time promote stable and prolonged treatment in the damaged tissue. The development of biocompatible controlled release delivery systems could increase treatment efficacy by allowing a lower dose of growth factor at the treatment site. Furthermore, the use of such systems would obviate the need for multiple injections of the protein to obtain a therapeutic effect.

In summary, certain important criteria that must be met before efficient neo-tissue growth is possible are [66]:

- Growth factors need to be delivered to the desired cell population thus ensuring that delivery to other cell types is avoided [67].
- The growth factors, upon injection *in vivo*, degrade rapidly and loose their bioactivity [67, 68].
- It is required that the tissue is continuously supplemented with growth factors for long periods of time allowing significant tissue repair [68].

In order to solve these requirements, controlled release polymeric delivery systems such as microparticles and nanoparticles **(Figure 2-2)** are currently available which offer many advantages over other immediate release delivery systems such as scaffolds [69].

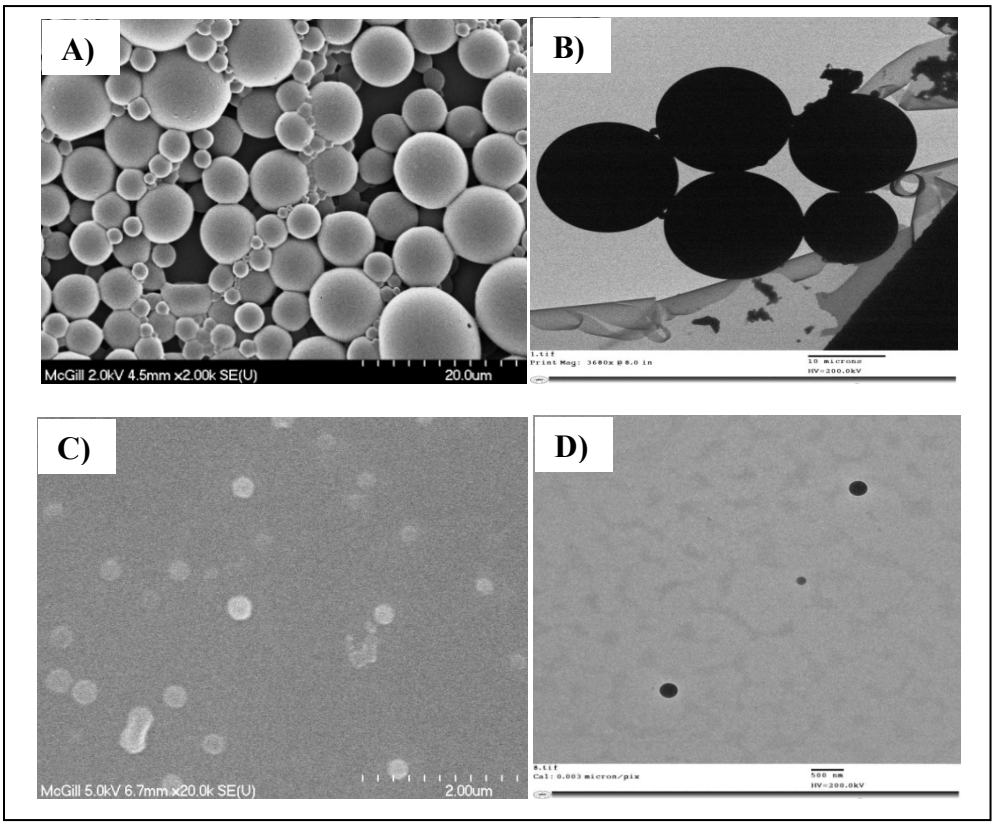

Figure 2-2: Microscopic Images of PLGA MPs and NPs
(A, C) Scanning electron microscope images **(B, D)** Transmission emission microscope images Scale Bar: 20 µm, 2µm **(A, C)** and 10µm, 500 nm **(B, D)**.

The advantages of using MP/NPs include:

- Reduced and prolonged dosing frequency
- Better therapeutic efficiency and control
- Fewer side effects

Although several groups have demonstrated the therapeutic angiogenic potential of using delivery vehicles loaded with several growth factors based on hydrogels, collagen-bound proteins or polymer scaffolds [70-73], such delivery systems are associated with drawbacks such as difficulty in controlling the release rate of growth factors from hydrogels and a strong initial burst release which in turn leads to severe side effects, such as hypotension. Furthermore, the use of MP/NPs which are easily injectable systems is additionally appealing as it removes the requirement for surgery to implant the drug delivery devices, such as scaffolds. In other words,

these MP/NPs allow minimally invasive delivery with efficient, convenient and infrequent dosing [74].

2.4.2 Factors controlling release rate of the delivery particles

The main focus while designing controlled drug release delivery particles is that the therapeutic agent should be delivered at the target site for an extended period of time, at a concentration within the therapeutic window so as to promote sufficient tissue regeneration [69]. The release of the therapeutic agent can be controlled by mechanisms such as diffusion, erosion, osmotic-mediated events or combinations of these [75, 76]. The release profiles of agents loaded within MP/NPs typically follow the same pattern of an initial burst release followed by a slower more sustained release constant over a certain period of time. The initial burst is basically due to the presence of drug precipitates at the surface of the particle, surface pores in the polymer and osmotic forces [75, 77]. The constant release of protein from the particles depends on the molecular weight and end-capping of the polymer [76]. Thus, the factors that could alter the release rate from a particle are:

- Reduction in the size of the particle (*i.e.*, an increase in the specific surface area) results in higher release [77].
- Particles with a higher porosity (i.e. a larger inner surface) have a higher drug diffusion rate due to increase in the influx of the release supernatant into the particles [78].
- Properties such as the chain length, flexibility and swelling behavior, potential interactions between polymer and drug also significantly affect the drug release rate [79, 80].
- The molecular weight and the use of an end group capped polymer also greatly control the diffusion and release rate of the drug [81].

2.4.3 Biomaterials used for formulation of MP/NPs:

The potential materials to be used for MP/NP synthesis should have certain essential characteristics to be considered suitable for drug delivery applications [82, 83]:

- Drug biocompatible
- Should be biodegradable with suitable degradation kinetics
- Should have suitable mechanical properties

- Ease of processing

Several polymers have been used for the development of MP/NPs including synthetic and natural polymers. Synthetic polymers provide advantages over the use of natural polymers such as a slow and continuous release of the therapeutic compound over several weeks. The application of synthetic polymers as therapeutic delivery devices for genes and drugs and imaging systems has drastically increased due to their desired properties of biocompatibility and biodegradability [84]. Synthetic polymers which have been used as drug- loaded systems include polyesters, polyamides, poly orthoesters, poly (amino acids), poly (alkyl-α-cyano acrylates), polyurethanes and polyacrlamides [85]. The aliphatic polymers such as poly glycolic and polylactic acid and essentially the copolymer poly-(lactic-co-glycolic acid) (PLGA) have been utilized extensively as biomaterials for several biomedical applications as early as the 1970s owing to their properties of biocompatibility and biodegradability [86, 87] and have in fact been approved for use in human therapeutic applications by the Food and Drug Administration (FDA).

2.4.4 Advantage of using PLGA MP/NPs and application in regenerative therapy

The most commonly used synthetic polymer with numerous biomedical applications, PLGA, is a copolymer of the two monomers, the cyclic dimmers (1, 4-dioxane-2,5-diones) of glycolic acid and lactic acid. Different forms of PLGA vary depending of the ratio of the monomers used such as PLGA 50:50 which has a composition of 50% lactic acid and 50% glycolic acid [88]. The main reason why PLGA MP/NPs are biodegradable is because they undergo hydrolysis after injection into the body through the formation of lactic acid and glycolic acid which are biocompatible and metabolizable moieties capable of easy removal from the host body through the Krebs cycle [89, 90]. The rate of degradation of the PLGA MP/NPs depends on the ratio of the monomers in the PLGA composition [91, 92]. For example, PLGA 50:50 containing equal ratio of lactic and glycolic acid would hydrolyze more rapidly in contrast to PLGA containing more amount of either one of the monomers.

PLGA MP/NPs have extensively been used for protein or growth factor delivery applications with successful and promising results highlighting the ability of these particles to promote tissue regeneration and repair both *in vitro* and in *in vivo* animal models. In a recent study by Formiga et al. the ability of VEGF loaded PLGA MPs to induce vasculogenesis and cardiac tissue remodeling was demonstrated in an acute myocardial ischemia-reperfusion model. The study

showed promising results and improved cardiac function allowing controlled delivery of active protein *in vivo* for more than a month after administration[18]. In another study, the use of PLGA NPs encapsulating VEGF again providing sustained delivery showed promise in increasing blood vessel growth for potential applications in tissue engineering[74]. Numerous studies have also shown the possibility of incorporating PLGA MP/NPs in polymeric scaffolds enabling a more controlled and extended release. Borselli et al. have recently showed that embedding PLGA MPs into a collagen matrix allowed preservation of the biofunctionality of the encapsulated protein in addition to allowing spatial and temporal tuming of the protein release pattern[93]. Another group demonstrated the potential of using bFGF loaded PLGA microparticles embedded with alginate scaffolds in promoting matrix vascularization after administration of the particles on the mesenteric membrane in rat peritoneum[94].

2.4.5 Preparation of PLGA MP/NP preparation:

PLGA nanoparticles are commonly synthesized using two techniques – emulsion solvent evaporation or through phase separation [91]. These techniques follow the same fundamental idea as during the synthesis of microparticles but involve slight variation in parameters leading to the formation of the nano-sized particles. The emulsion solvent evaporation method can be further divided into single emulsion process involving oil-in-water (o/w) emulsification or double emulsion process involving water-in-oil-in-water (w/o/w) emulsification. The technique for MP/NP preparation basically depends on the application. For instance, in the case of water soluable drugs like proteins, peptides and vaccines, the w/o/w method is best suited while o/w method is best suited for water-insoluble drugs like steroids.

In the single emulsion technique (o/w), the polymer, example PLGA, is dissolved in an organic solvent, example dichloromethane (DCM) followed by addition of the therapeutic agent to this polymer-organic solvent solution leading to the formation of a dispersion of drug particles [91]. This polymer-organic solvent-therapeutic agent dispersion is then emulsified in the presence of an emulsifier, such as the commonly used poly-(vinyl alcohol) (PVA), under appropriate temperature conditions and stirring. The last step involves removal of the solvent from the MP/NPs either by extraction or evaporation and collection by filtration, centrifugation or sieving. Finally, the MP/NPs are obtained either by lyophilization or drying under suitable conditions depending on the encapsulated therapeutic agent [91].

In the double emulsion technique (w/o/w), aqueous solution of the therapeutic agent to be loaded into the particle is added to the organic phase, formed by dissolving the polymer into the organic solvent such as DCM. The water-in-oil emulsion so formed is then either stirred or homogenized and then added to a solution of emulsifying agent dissolved in water such as PVA thus forming the water-in-oil-in-water emulsion. Finally the solvent in lastly removed [91]. **(Figure 2-3)** provides a schematic representation of the emulsification-solvent evaporation method for particle preparation.

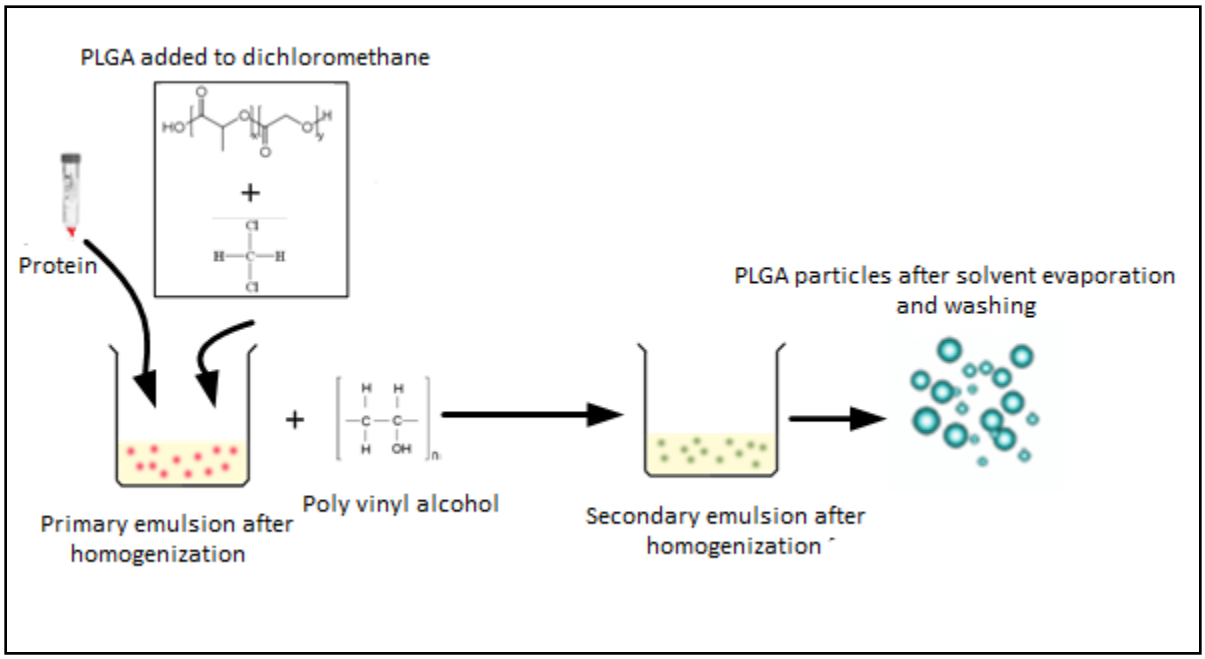

Figure 2-3: Schematic representation of the emulsification-solvent evaporation method for particle preparation.

The double emulsion solvent evaporation technique can be used to encapsulate therapeutic proteins and peptides into polymeric particles [85]. However, the main concern with protein encapsulation in PLGA MP/NPs is the loss of therapeutic efficacy due to the protein degradation or denaturation. Protein inactivation could take place basically due to two mechanisms. The first being due to protein exposure to organic solvents during particle preparation, which leads to protein adsorption at the oil–water interface and causes protein denaturation and aggregation [95, 96]. The degradation of PLGA matrix causes an acidic environment which also leads to inactivation of the protein [97]. Studies have shown that addition of bovine serum albumin (BSA) to the aqueous phase before emulsification protects the therapeutic protein from

aggregation as BSA preferentially absorbs to the particle interface[96]. Other studies have also demonstrated that addition of a buffering base such as magnesium hydroxide to the PLGA microsphere formulation was shown to protect the encapsulated BSA from aggregation and degradation due to the acidic environment and increase its *in vitro* release from the microspheres[97].

In the phase separation technique, the polymer is first dissolved in the organic solvent followed by the addition of the therapeutic agent to this polymer-organic solvent solution [91]. Then, another organic solvent (excluding the organic solvent taken initially) is then added to the polymer solution under constant stirring such that the polymer solvent is extracted; leading to the creation of tiny coacervate droplets of the drug loaded polymer which size depending on the stirring speed during formation. The particles are then extracted either through centrifugation or through filtration.

Before administration into the host it is essential that the necessary sterilization steps are taken to reduce the risk of infection and immune rejection [98]. The most commonly used method for sterilization of PLGA particle systems is through the use of ϒ-irridiation. However, this technique has drawbacks such as instability, deterioration of the polymer and breakage of the cross-linking polymer chains. Several studies are being carried out to design the ideal method of sterilizing PLGA biodevices without the damaging effect of structural changes to the polymer but it has been shown that more research has to be done before such as technique can be optimized. Yet, from their studies, Shearer et al. indicated that antibiotic treatment could be an effective and easy method for the sterilization of PLGA fibers during scaffold development in tissue engineering applications [99, 100].

2.5 Research goal and present approach

The use of adult stem cells in regenerative therapy has been investigated in depth by several researchers. This regenerative effect created by injected stem cells has been attributed to several mechanisms which include differentiation, cell fusion and the release of growth factors by the cells or paracrine effects[52]. However, there has been an increase in evidence by numerous groups supporting the hypothesis that the regenerative and healing effect promoted by the administered stem cells could actually be due to the paracrine mechanisms or growth factors

secreted by the stem cells[8, 10]. Other groups have also demonstrated that the administration of pro-angiogenic proteins promotes tissue regeneration, but these results have shown limited success in a clinical setting due to the denaturation and short lived effect of the protein when administered as a bolus[17]. With studies showing successful results achieved through the administration of stem cell-derived growth factors for potential application in tissue regeneration therapy, we believe that the use of a controlled delivery particle system allowing sustained and protected release of the encapsulated stem cell derived growth factor cocktail could augment the potential of this strategy. In our first study, we first combined two recombinant pro-angiogenic proteins available in the market to understand and observe the mitotic and anti-apoptotic effect of using a combination of growth factors instead of individual proteins, co-encapsulated inside polymeric nanoparticles on human umbilical vein endothelial cells (HUVECs). *In vitro* release studies were done to observe the release profiles of the two proteins from the nanoparticles. In the second study, we move on to the main goal of the thesis, which was to efficiently harness the growth factor cocktail from bone marrow stem cells and load the concentrated protein cocktail into polymeric nanoparticles. *In vitro* studies were done to observe the release profile of a key pro-angiogenic protein, VEGF, present in the stem cell factor cocktail released from the particles. This was followed by functional activity studies of the released factors from the particles using angiogenic assays with endothelial cells to confirm that the released growth factors remained active even after the particle preparation process. Furthermore, to assess the potential of this concept for possible application in myocardial repair, an *in vivo* rat model of MI was used to analyze the effect and efficiency of the factors released from the particles into the myocardium, there by mimicking the stem cell paracrine mechanism. The final study involved embedding stem cell derived factor cocktail loaded microparticles into a hydrogel with the aim of creating a system which allowed a more localized, controlled and extended release of the protein cocktail for a more prolonged therapeutic effect at the target site for possible tissue regeneration and wound healing applications. The stem cell cocktail was generated from adipose derived stem cells which are easier to isolate and culture in comparison to bone marrow derived stem cells. *In vitro* experiments involved release studies of two key pro-angiogenic proteins present in the cocktail, VEGF and bFGF, followed by biofunctionality assessment of the factors released from the composite system using endothelial cells.

3 Preface for Chapters 4, 5 and 6

Chapters 4, 5 and 6 present the results obtained during this research project. In chapter 4, our aim was to understand the mitotic and anti-apoptotic effect of using a combination of key pro-angiogenic proteins instead of individual proteins loaded into a polymeric particle system. Recombinant hVEGF and hAng-1, available freely in the market, were coencapsulated in NPs and experiments were performed to observe the different release profiles of individual proteins from a protein mixture loaded into the nanoparticles followed by assessment of functional activity of the released proteins. Chapter 5, provides the proof of concept of the main goal of the thesis for the strategy of using controlled delivery polymeric systems encapsulating stem cell derived growth factors. Growth factors released from bone marrow derived stem cells (hBMSCs) under hypoxic conditions were encapsulated into NPs with *in-vitro* studies to observe the release profiles and bioactivity of key proteins in the cocktail released from the NPs followed by *in-vivo* analysis. Chapter 6 demonstrates the possibility of localized, extended and protected release of the encapsulated stem cell cocktail by embedding the delivery particles into a hydrogel. The potential of using stem cell cocktail harnessed from easily accessible adipose stem cells (hASCs) and loaded into MPs and embedded into a polymeric hydrogel for possible tissue and wound healing applications was investigated. *In vitro* studies were done to observe the release profile of two key pro-angiogenic proteins present in the cocktail followed by biofunctionality studies of the released cocktail.

Research Articles Presented in the Thesis Chapters 4, 5 and 6:

1) **Khan A A,** Paul A, Abbasi S, Prakash* S. "Mitotic and antiapoptotic effects of nanoparticles coencapsulating human VEGF and human angiopoietin 1 on vascular endothelial cells." (International Journal of Nanomedicine, Vol. 2011:6, 2011)

2) **Khan A A,** Paul A, Prakash* S. "Controlled delivery of bone marrow stem cell-derived growth factors using polymeric nanoparticles improves myocardial repair and function in **i**nfarcted rat heart model"

3) **Khan A A,** Paul A, Prakash* S. "Controlled delivery of adipose stem cell derived condition media using polymeric microparticles embedded in hydrogel for effective angiogenic therapy: preparation and *in vitro* analysis"

Contribution of Authors:

I am the first author for all the above articles and was responsible for the research design, procedure and data analysis. All the other authors have also contributed significantly by providing suggestions and assistance in performing experiments and are reported as co-authors. The last author, Dr. Satya Prakash, is the research advisor and is also the corresponding author in all of the manuscripts, abstracts and proceedings written throughout the course of the current Masters project.

Review Article:

1) Prakash* S, **Khan A**, Paul A. "Nanoscaffold based stem cell regeneration therapy: recent advancement and future potential" (Expert Opinion on Biological Therapy, Vol. 10, 2010)

Contributions from the Current Research not included in the Thesis:

1) **Khan A**, Paul A, Abbasi S, Prakash* S. "Nanoparticles coencapsulating human VEGF and human angiopoietin-1 can induce mitotic and antiapoptotic effects on vascular endothelial cells." TechConnect World 2011, Boston

Contributions to research un-related to current research:

During my master's studies, I had the opportunity to learn and contribute to other research. I am a co-author in the following papers:

1) Paul A, **Khan A A**, Shum-Tim D, Prakash* S. "BacMam Virus Transduced Cardiomyoblasts Can Be Used for Myocardial Transplantation Using AP-PEG-A Microcapsules: Molecular Cloning, Preparation, and In Vitro Analysis" (Journal of Biomedicine and Biotechnology, Vol. 2010, 2010)
2) Paul A, Binsalamah Z, **Khan A A**, Abbasi S, Cynthia B. Elias, Shum-Tim D, Prakash*S. "A novel nanobiohybrid complex of recombinant baculovirus and Tat/DNA nanoparticles for efficient gene delivery: implication in myocardial infarction therapy using *Ang-1* transgene" (Biomaterials, Vol. 32, 2011)
3) Paul A, Chen C, **Khan A A**, Rao V, Shum-Tim D, Prakash*S. "Genepin-crosslinked microencapsulated adipose derived stem cells augment transplantation retention resulting in attenuation of chronically infarcted rat heart fibrosis and cardiac dysfunction" (Cell Transplation, 2011)
4) Binsalamah Z, Paul A, **Khan A A,** Prakash S, Shum-Tim D. " Intramyocardial sustained delivery of placental growth factor using nanoparticles as a vehical for delivery in the rat infarct model" (International Journal of Nanomedicine, Vol. 2011:6, 2011)

5) Abbasi S, Paul A, **Khan A A**, Shao W, Malhotra M, Prakash* S. "HIV-1 TAT peptide surface functionalized albumin nanoparticles for improved gene delivery: optimization of *in vitro* transfection conditions to target breast cancer." (Under consideration, 2011)
6) Abbasi S, Paul A, Shao W, **Khan A A**, Prakash* S. "Cationic albumin nanoparticles for enhanced drug delivery to treat breast cancer: preparation and *in vitro* assessment." (Under consideration, 2011)
7) Coussa-Charley M, Marinescu D, Paul A, **Khan A A**, Prakash* S. "*Lactobacillus.* Probiotic can efficiently hydrolyze bile salts and displace potential pathogen in a simulated gut model". (To be submitted to Journal of Biologics: Targets and Therapy, 2011)
8) Coussa-Charley M, Rodes L, Paul A, Fakhoury M, Al-Salami H, Abbasi S, **Khan A A**, Prakash* S. "A novel continuous gut adhesion model using a packed bed bioreactor". (Submitted to Biotechnology Research International, 2011)
9) Coussa-Charley M,Rodes L, Paul A,Fakhoury M, Al-Salami H, Abbasi S, **Khan A A**, Prakash* S. "Investigation of the effect of *Lactobacillus* probiotic strain addition on the intestinal microflora immobilized on a novel continuous gut adhesion model". (To be submitted to Journal of Biologics: Targets and Therapy, 2011)
10) Paul A, Nayan M**, Khan A A**, Shum-Tim D, Prakash* S. "Hybrid nanocomplex of baculovirus and TAT/DNA nanoparticles as a novel gene delivery vehicle for adipose stem cell-gene therapy: functional assessment in myocardially infarcted rat model". (Under Preparation, 2011)
11) Rodes L, **Khan A A**, Coussa-Charley M, Paul A, Shao Wei, Prakash* S. " *In vitro* screening of anti-inflammatory properties of probiotics using a human colonic microbiota model and RAW 264.7 macrophage cells" (Under preparation, 2011)

Research abstracts:

1) Paul A, **Khan A**, Shum-Tim D, Prakash S. Microencapsulated genetically modified stem cells for heart tissue regeneration. Second World Congress of International Acadamy of Nanomedicine (IANM), Antalya, Turkey, 2010.
2) Binsalamah Z, Paul A, Cantor A, **Khan A**, Prakash S, Shum-Tim D.The Application of Microcapsules and Nanoparticles in Cellular Cardiomyoplasty. Presented at the Chongqing International Heart Congress, China, April 23, 2011.
3) Binsalamah Z, Paul A, Cantor A, **Khan A**, Prakash S, Shum-Tim D. Novel Cardiovascular Devices: The Efficacy of Using Microencapsulation and Nanoparticles for Cell-based Therapy in Myocardial Regeneration. To be presented at the Annual Meeting of the Canadian Society of Clinical Perfusion, Vancouver, BC, October 23, 2011.
4) Paul A, Cantor A, Abbasi S, **Khan A**, Shum-Tim D, Prakash S. Biocompatible Polymeric Microcapsules: A Novel Delivery System for Efficient Myocardial Delivery of Human Mesenchymal Stem-cells. Canadian Cardiovascular Congress 2010, Montreal.
5) Abbasi S**,** Paul A, **Khan A**, Prakash S. "Delivery Using Biodegradable Nanoparticles for Breast Cancer Therapy." TechConnect World 2011, Boston

4 Mitotic and antiapoptotic effects of nanoparticles co-encapsulating hVEGF and hAng-1 on vascular endothelial cells

Research article

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4.1 Preface

In the previous chapter, an introduction to the need and benefits of using controlled delivery particle systems for biologically active molecules such as proteins, for potential application in regenerative therapy is given. In this chapter, our aim was to understand the mitotic and anti-apoptotic effect of using a combination of key pro-angiogenic proteins encapsulated into a polymeric particle system. Recombinant hVEGF and hAng-1, available freely in the market, were coencapsulated in human serum albumin (HSA) NPs. The HSA NPs were first characterized by measuring the particle size, surface zeta potential and morphology followed by cytotoxicity studies where an optimal concentration of glutaraldehyde for NP coating process was determined to develop stable and less toxic NPs as protein carriers. The HSA NPs were then incubated for two weeks to observe the release profiles of the proteins from the NPs. To check if the functional activity of the released proteins had been retained, angiogenic assays using endothelial cells were performed.

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4.2 Abstract

Research towards the application of nanoparticles (NPs) as carrier vehicles for the delivery of therapeutic agents is increasingly gaining importance. Angiogenic growth factors, human vascular endothelial growth factor (hVEGF) and human Angiopoietin-1 (hAng-1) are known to prevent vascular endothelial cell apoptosis and in fact stimulate human vascular endothelial cells (HUVECs) proliferation. This paper aims to study the combined effect of these bioactive proteins, coencapsulated in human serum albumin (HSA) NPs, towards HUVECs and evaluate the potential application of this delivery system towards therapeutic angiogenesis. The angiogenic proteins hVEGF and hAng-1 were coencapsulated in albumin NPs for a better controlled delivery of the proteins. The application of a NP system enabled efficient and extended release kinetics of the proteins. The size of the NPs cross-linked with glutaraldehyde varied between 101.0$\pm$ 0.9 nm and the zeta potential was found to be -18$\pm$ 2.9 mV. An optimal concentration of glutaraldehyde for NP coating process was determined which provided stable and less toxic NPs as protein carriers. Results of the study indicate that NPs cross-linked with glutaraldehyde produced NPs with tolerable toxicity which provided efficient and controlled release of the coencapsulated proteins. The NPs were incubated for two weeks to determine the release profiles of the proteins. At the end of the two week incubation period, it was observed that 49$\pm$1.3 % of hAng-1 and 59$\pm$2.1 % of hVEGF had been released from the NPs.

The proliferation and percent apoptosis of HUVECs in response to the released proteins was observed. The results indicate that the released proteins were biologically active and the combined application of both the proteins demonstrated a significantly high proliferatory and antiapoptotic effect on HUVECs as compared to the effect demonstrated by the released individual proteins. These studies could serve as a basis to encourage further research into the potential *in vivo* application of these protein loaded NPs in the field of therapeutic angiogenesis.

Keywords: Growth factors, encapsulation, nanoparticles, nano-biotechnology, angiogenesis, regenerative medicine

4.3 Introduction

Vasculogenesis and angiogenesis are the two essential mechanisms that implement the development of the vascular network [101, 102]. Vasculogenesis is a process that occurs during

early development of an individual during blood vessel network formation whereas angiogenesis which is a similar process does not occur only at the time of the creation but is a mechanism that occurs throughout the organism's life. Angiogenesis is basically associated with the proliferation, migration and remodelling of fully differentiated endothelial cells and involves sprouting of new smaller blood vessels from pre-existing ones and the repair of damaged blood vessels at the site of an injury. It is known that programmed cell death or in other words apoptosis is required for the normal development of multicellular organisms where unwanted cells are eliminated during physiological and certain pathological conditions [103]. However, it has also been shown that the dysregulation of endothelial cell apoptosis has a major regulatory effect on the establishment of the primordialvascular network termed vasculogenesis in the embryo causing severe haemorrhage and finally leading to embryonal death. Counteracting proliferation, endothelial cell apoptosis in excess may limit angiogenesis thus leading to vessel regression. It is hence evident that the inhibition of endothelial cell apoptosis can serve as a potential therapeutic target, especially in the patients suffering from ischemic diseases where prevention of apoptosis could improve angiogenesis and vasculogenesis.

Several growth factors and proteins have demonstrated the ability to prevent vascular endothelial cell apoptosis and in fact stimulate endothelial cell proliferation. Vascular endothelial growth factor (hVEGF) is a mitogenic and chemotatic factor for endothelial cells and seems to play a vital role in the protection of these cells against apoptosis [104-106]. The use of hVEGF as a potential stimulant in therapeutic angiogenesis has been widely demonstrated [107-114]. However, there still lies an uncertainty in whether the presence of hVEGF alone would suffice in the achievement of functional and mature vessels lined with vascular smooth muscles or pericytes. There is evidence that excessive hVEGF expression could bring about pathological and immature vessel formation, enhanced vascular wall permeability and could lead to angioma formation [45, 115, 116]. In this context, research has shown that the ligand of Tie2 receptor, Angiopoietin-1 (hAng-1), has the ability to stabilize and assist in the maturation of blood vessels [117-119] and in fact abrogates endothelial cell apoptosis [120, 121]. However, there has been debate over the role of hAng-1 as an endothelial cell mitogen. While some studies have demonstrated hAng-1 to be a potent mitogen of endothelial cells [122], others have pointed out that hAng-1 either failed to induce proliferation [123] or did not cause significant increase in the proliferation [124]. As a whole however, the administration of combination of hAng-1 with

hVEGF may prove to be an efficient strategy for therapeutic angiogenesis. Moreover, several studies have also demonstrated that application of multiple growth factors for therapeutic applications has a greater impact as compared to single growth factor delivery [125-127].

Extensive research is being carried out to discover the potential of using recombinant proteins towards therapeutic applications. However, due to their very limited in vivo half-lives, the proteins have to be administered through multiple injections so as to achieve the desired therapeutic effect. These macromolecules also have high molecular weights which lead to low bioavailability, have high aqueous solubility, and are incapable of diffusing through biological membranes [128, 129]. It is thus evident that appropriate delivery vehicles are developed for these macromolecules, which are in fact very unstable, so as to protect them from degradation in the biological environment of the organism. The potential of using these molecules towards therapeutic applications greatly depends on the use of a suitable vehicle for delivery. Thus, numerous strategies have been applied towards the design of appropriate vehicles for efficient sustained growth factor delivery [130]. However, the use of NPs as protein delivery systems has gained interest. The use of NPs has several advantages over the microparticle encapsulation [85]. By encapsulating the macromolecules, the physicochemical properties of the particles allow a more controlled and continuous protein release over a period of several weeks.

Amongst the existing delivery systems, NPs based on natural biopolymers have shown to be very promising as compared to those made of synthetic materials. Albumin is a physiological protein that is found in abundance in the blood and can be purified easily. In our study we made use of human serum albumin (HSA) as the developing material for the delivery system. HSA has a molecular weight of 66 kDa and has the ability to bind to hormones, long chain fatty acids, metal ions with high affinity and vitamins thus serving as a transport and depot protein for a variety of compounds [131]. Studies have also shown that HSA binding results in decreased toxicity and an increase in the in-vivo half life of the bound ligands [132]. In literature, there exist three methods for the preparation of HSA based NPs based on emulsion formation, desolvation, or coacervation processes [133-135]. In order to stabilize the resulting NPs, glutaraldehyde can be used as the cross-linking agent [135, 136]. However, to date the application of NPs towards the encapsulation of growth factors is lacking. The objective of this study was to investigate the

feasibility of entrapping the growth factors, hVEGF and hAng-1, in NPs cross-linked with glutaraldehyde to enable potential application in therapeutic angiogenesis.

4.4 Materials and Methods

4.4.1 Materials

Human serum albumin (HSA fraction V, purity 96-99%), Bovine serum albumin (BSA), guanidine hydrochloride, 8% glutaraldehyde, were purchased from Sigma Aldrich (Oakville, Ontario, Canada). The human vascular endothelial growth factor (hVEGF), human Angiopoietin1 (hAng-1), and their respective ELISA kits were purchased from R & D systems (Minneapolis, Minnesota, USA), CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation MTS Assay kit (Promega, Madison, Wisconsin, USA) was to detect cytotoxicity and cell proliferation. Antibodies against hVEGF and hAng-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA).

4.4.2 Cell culture

Human Umbilical Vein Endothelial Cells [HUVEC] (Sciencell, Carlsbad, California, USA) were cultured and expanded on tissue culture plates according to the supplier's instructions. They were cultured in Endothelial cell medium (ECM) (Sciencell, Carlsbad, California, USA) supplemented with 5% (v/v) fetal bovine albumin (FBS) and placed in an incubator containing 5% CO_2 at 37°C. The cells used in this study were from between passages 2 and 5.

4.4.3 Preparation of the NPs

As demonstrated by our group earlier, HSA NPs were prepared using pH-coacervation technique [137]. Briefly, 100mg of HSA protein was added to 2ml 10mM NaCl solution in a glass vessel under constant stirring at room temperature. The pH was brought to 8 by the addition of 0.1N NaOH. Then, 14µl of 0.5mg/mL of hAng-1, 1µl of 0.5mg/mL of hVEGF and 1µl of 0.5mg/mL of BSA dissolved in ECM (with or without 5% FBS) was added into the above solution and this aqueous phase was then desolvated with drop wise addition of 4ml of ethanol at a constant rate of 1ml/min to form the NPs. The concentration of protein added during the NP preparation was optimized based on the fact that when the inhibitory doses of hAng-1 was almost ten times more than hVEGF, both caused identical antiapoptotic effects in HUVECs and using a combination of hAng-1 with hVEGF produced a significantly higher antiapoptotic effect [121]. NPs containing BSA were taken as negative control. The protein encapsulated NPs formed were then stabilized

by coating with 40µl of 5% glutaraldehyde. The coated NPs were purified by ultracentrifugation at 20,000g for 15 minutes and the pellet was redispursed into ddH_2O to original volume. The purification process was carried out three times with each redispersion step performed in an ultrasonication bath, Bransonic® Ultrasonic Cleaner 2510 (Branson, Danbury, Connecticut, USA) for 15 minutes. The entire procedure of particle preparation was carried out under sterile conditions and all the solutions used during preparation were sterilized by passing through 0.20µm sterile filters before use. The encapsulation efficiency of both the proteins was determined in terms of the amount of hAng-1 and hVEGF transformed into NPs before coating using hAng-1 and hVEGF ELISA kits. The NPs were centrifuged at 20,000g for 15 minutes at room temperature to separate the NPs from the supernatant. Percentage encapsulation efficiency was calculated as:

$$\left(\frac{\text{initial amount of growth factor} - \text{growth factor amount in the supernatant}}{\text{initial amount of growth factor}}\right) \times 100$$

4.4.4 Particle size and zeta potential of NPs

The particle size, zeta potential and polydispersity index (PDI) of the NPs was measured by the technique of electrophoretic laser Doppler anemometry by using a Zeta Potential Analyzer (Brookhaven Instruments Corporation**,** HotsVille, New York, USA). For zeta potential determination, 0.005M phosphate buffer with pH 7.0 was used. ZetaPlus Particle Sizer Software Version 4.11 was used to determine the size distribution of the NPs and Zeta Potential Analyzer Version 3.57 was the software used for the zeta potential. Both the particle size and the zeta potential were measured for three batches of NPs and each measurement was obtained after taking the average of the three runs.

4.4.5 NP surface characterization

Scanning Electron Microscopy (SEM) was used to study the morphological characteristics of the NPs. The NPs were dispersed in ddH_2O and then dried with natural convection under room temperature. The samples were then examined under S4700 FEG-SEM (Hitachi, Oakville, Ontario, Canada).

Transmission Electron Microscopy (TEM) was used to obtain the size characterization. The NPs were suspended in 1X PBS and analyzed on CM200 FEG-TEM (Philips, Markham, Ontario, Canada).

For the Atomic Force Microscopy (AFM) measurements the NP samples were sonicated for 5 minutes and 1µl of the sample was dropped on to the surface of the discs and dried using conventional method. Images were produced with Nanoscope III AFM (Digital Instruments, USA) using silicon cantilever in tapping mode and analyzed using Nanoscope software version 5.12r5.

4.4.6 In vitro cytotoxicity of NPs with MTS assay

CellTiter 96® AQueous Non-Radioactive Cell Proliferation MTS Assay kit was used to assess the in vitro cytotoxicity of the various concentrations of glutaraldehyde on HUVECs [137]. The HUVECs were seeded at a density of $2x10^4$ cells/well in a 96 well plate in fresh medium. Twenty-four hours post seeding, the HUVECs were washed with PBS and incubated with NPs cross-linked with 40µl of 1.5%, 3%, 4.5%, 6% and 7.5% and control NPs without glutaraldehyde cross-linking for 96 hours. The relative HUVEC viability was determined by MTS assay which was performed as per the manufacturer's protocol. Briefly, after 96 hours of incubation, the HUVECs were washed with PBS and fresh ECM was added to the wells. This was followed by the addition of 20µl of MTS solution to each well and the cells were incubated for 4 hours at 37°C The measurement of the absorbance was carried out using the 1420-040 Victor3™ Multilabel Counter (Perkin Elmer, Woodbridge, Ontario, Canada) at 490nm. An optimal concentration of glutaraldehye was chosen to form the coating of the protein loaded NPs.

4.4.7 In vitro release of co-encapsulated hAng-1 and hVEGF from NPs

The protein loaded NPs were coated with 40 µl of 5% glutaraldehyde for this study. The NPs were incubated with 5ml of ECM with 1% penicillin/streptomycin at 37°C under constant shaking. The experiment was carried out in triplicates. The samples were then centrifuged at 20,000g for 15 minutes at the indicated time points and 0.4 ml of supernatant was removed. In order to maintain a constant volume the same amount of fresh ECM was added back to the pellet. The amount of hAng-1 and hVEGF protein released into the supernatant was determined using hAng-1 and hVEGF ELISA kits, respectively, according to manufacturer protocols.

4.4.8 HUVEC proliferation assay

The bioactivity of released hVEGF and hAng-1 dissolved in ECM (supplemented with 5% FBS) from the NPs was evaluated in vitro by determining the proliferative capacity of HUVECs. CellTiter 96® AQueous Non-Radioactive Cell Proliferation MTS Assay kit was used to assess

the proliferation of the HUVECs treated with NP supernatant [138, 139]. The HUVECs were plated in a density of $2x10^4$ cells/well in a 96 well plate in ECM containing 5% (v/v) FBS. After 24 hours, the ECM was removed and the cells were then incubated for 96 hours with 0.1µl of NP supernatant, NP supernatant with excess hVEGF antibody and NP supernatant with excess hAng-1 antibodyreleased from the NPs into the ECM on day 2, 8 and 14. The results from the ELISA were used to determine the concentration of hAng-1 and hVEGF in the NP supernatant. Control samples received same amount of ECM (supplemented with 5% FBS) and NP supernatant with BSA. The relative HUVEC viability was determined by MTS assay which was performed as per the manufacturer's protocol. Briefly, after 96 hours of incubation, the HUVECs were washed with PBS and fresh ECM was added to the wells. This was followed by the addition of 20µl of MTS solution to each well and the cells were incubated for 4 hours at 37°C The measurement of the absorbance was carried out using the 1420-040 Victor3^{TM} Multilabel Counter (Perkin Elmer, Woodbridge, Ontario, Canada) at 490nm.

4.4.9 HUVEC apoptosis assay

To determine the percentage apoptosis, NPs were prepared using the same particle preparation protocol as before but the proteins were dissolved in serum-free ECM (without 5% FBS) instead of ECM with 5% FBS and then added to the HSA solution. The NPs obtained were then incubated with 5ml of serum-free ECM with 1% penicillin/streptomycin at 37°C, with constant shaking. The samples were then centrifuged at the indicated time points and 0.4 ml of supernatant was removed. In order to maintain a constant volume the same amount of fresh serum-free ECM was added back to the pellet. HUVECs were then seeded onto a 96-well plate at a cell density of $2x10^4$ cells per well in ECM supplemented with 5% FBS and incubated at 37°C for 24 hours. After the incubation period, the cells were then washed three times with PBS and the medium was then replaced with 0.1mL of NP supernatant, NP supernatant with excess hVEGF antibody, NP supernatant with excess hAng-1 antibody from day 2, 8 and 14, and incubated for 96 hours. Control cultures received the same amount of serum-free ECM without the addition of hVEGF and hAng-1 proteins and NP supernatant containing BSA. The numbers of cells floating in each well were collected after PBS washing and counted. The number of apoptotic cells in the adherent cells was then determined after 96 hours using MitoTracker® Red CMXRos kit and DAPI nucleic acid staining (Invitrogen, Burlington, Ontario, Canada), according to the manufacturer's instructions, and were counted from random locations in the

well using fluorescence microscope [140]. Both the staining techniques gave similar results and the apoptosis was demonstrated as the percentage of apoptotic adherent cells and floating cells in a well. The experiment was carried out in triplicates.

4.5 Results and Discussion

4.5.1 Characterization of NPs

In this study, the NPs were prepared by a pH-coacervation technique followed by the glutaraldehyde cross-linking. Coacervation agents such as ethanol are widely used in protein purification processes and NPs are prepared using coacervation methods by the addition of coacervation agents to the protein solution [141]. The NPs were prepared by adjusting the pH of the HSA solution to 8.0 and controlling the amount of coacervation agent to form NPs of size between 101.0$\pm$ 0.9 nm, shown in **(Figure 4-2A)**. It is known that the size of the resultant NPs depends on the amount of the coacervation agent but not on the amount of the glutaraldehyde added or the cross-linking procedure [135].

The TEM, SEM and AFM micrographs of the NPs illustrate that the particles are properly dispersed, spherically shaped and have smooth surface characteristics as shown in **(Figure 4-1)**. **(Figure 4-1A)** is the TEM image of the NPs before ultrasonication which indicates that before ultrasonication the NPs form clusters and are not properly dispersed. **(Figure 4-1B)** provides the TEM image of the NPs after ultrasonication for 15 minutes. Here it is demonstrated how ultrasonication assists in separating out the particles more uniformly and the particles are properly dispersed. **(Figure 4-1C)** gives the TEM image of the NPs after magnification. **(Figure 4-1D)** is the SEM image for the NPs cross-linked with 40µl of 5% (w/v) glutaraldehyde which demonstrates the smooth surface characteristics of the NPs. **(Figure 4-1E)** provided the three-dimensional AFM image of the NPs.

The surface charge and the size of the particles are the two main surface characteristics that influence the biodistribution of the NPs upon administration. The zeta potential of the glutaraldehyde cross-linked NPs decreases when the concentration of glutaraldehyde is increased [135]. Zeta potential measurements were performed in phosphate buffer at pH 7.0 as pH and ionic strength of the dilution medium affects the magnitude of the zeta potential. The value was found to be in accordance with the results shown by Lin et al [141]. The protein NPs were

negatively charged and the zeta potential varied between -18± 2.9 mV at pH 7.0 as shown in **(Figure 4-2B)**. The polydispersity index was found to be 0.3.

4.5.2 Determination of cytotoxic effects of NPs on HUVECS

Glutaraldehyde has been widely used as a cross-linking agent in the preparation of NPs. However, it is known to induce cytotoxicity and higher concentrations of glutaraldehyde in particle preparation can lead to an increased toxicity to the cells. Therefore, an optimal amount of glutaraldehyde to be used in the particle preparation was determined for stable and less toxic NPs. The cytotoxicity of the blank NPs cross-linked with a series of glutaraldehyde concentrations was studied by incubating HUVECs for 96 hours with the NPs. MTS assay was used to determine the percentage of viable cells compared to the initial number of cells. As shown in **(Figure 4-3)**, HSA-NPs without glutaraldehyde coating showed a cell viability of above 90% after 96 hours. Increasing the concentration of glutaraldehyde in the particle preparation increased the cytotoxicity of the NPs to the seeded HUVECs. 40µl of 5% (w/v) glutaraldehyde was chosen as the optimal concentration for the NP preparation which resulted in the formation of stable and uniform particles suitable for protein delivery. It should be noted that in the future, further research might be necessary to replace the application of glutaraldehyde with more biocompatible polymers, but for this study, NPs cross-linked with glutaraldehyde loaded with hAng-1 and hVEGF were further studied to investigate if the bioactivity of the proteins was maintained.

4.5.3 Encapsulation efficiency and release kinetics profile of proteins co-encapsulated in NPs

It has been shown that coating the NPs with a cross-linking agent increases the encapsulation efficiency, reduces the initial burst release and extends the protein release period to a slower more efficient release [142]. In our studies, 40µl of 5% glutaraldehyde was taken as the optimal amount as cross-linking agent producing stable NPs with a reduced initial burst release and slower and steady extended release profiles for both hAng-1 and hVEGF. The encapsulation efficiency of hAng-1 and hVEGF during NP fabrication was 49% and 55% respectively. Cumulative release kinetics studies were conducted over 2 weeks to determine the release of hAng-1 and hVEGF from the NPs in the culture medium. The release kinetics profiles of both the proteins from the NPs are illustrated in **(Figure 4-4)**. A burst release of both the proteins was observed within the initial two days followed by a lower steady release phase. The burst release

amounts for hAng-1 and hVEGF were approximately $617 \pm 100\, ng$ and $88 \pm 10\, ng$, respectively, with lower steady release amounts of approximately 171.5 ng and 13.75 ng for hAng-1 and hVEGF, respectively. At the end of the two week incubation period it was observed that 49% of hAng-1 and 59% of hVEGF had been released from the NPs. The cumulative release amounts for hAng-1 and hVEGF on day 14 were $1680.7 \pm 150\, ng$ and $162.25 \pm 17\, ng$, respectively.

4.5.4 Retention of Bioactivity of released hAng-1 and hVEGF on HUVECs

hVEGF is an endothelial cell (EC)-specific mitogen, which enhances vascular permeability, induces endothelial cell proliferation and stimulates angiogenesis in vivo [107-114]. However, as discussed earlier, there has been debate over the role of hAng-1 as an endothelial cell mitogen. While some studies have demonstrated hAng-1 to be a potent mitogen of endothelial cells [122] others have pointed out that hAng-1 either failed to induce proliferation [123] or did not cause significant increase in the proliferation [123]. Our results indicate that the hVEGF in combination with hAng-1 released from the NPs in fact helps to significantly augment HUVEC proliferation which could enable the potential application of this delivery system for therapeutic angiogenesis.

The bioactivity of the released hVEGF, hAng-1 and BSA in media from the NPs was evaluated in vitro by observing the proliferative capacity of the HUVECs. CellTiter 96® AQueous Non-Radioactive Cell Proliferation MTS Assay kit was used to assess the proliferation of the HUVECs treated with NP supernatant. The results were illustrated as the percent increase in cell proliferation relative to the unstimulated control. As shown in **(Figure 4-5)**, the least cell proliferation occurs with NP supernatant containing hAng-1 (hVEGF antibody added), moderate proliferation with NP supernatant containing hVEGF (Ang-1 antibody added), and finally the highest proliferation is observed in the case of NP supernatant containing both hVEGF and hAng-1 combined. As expected, the control NP supernatant containing BSA produced negligible results. The effect of supernatant containing Ang-1 on cell proliferation is relatively low compared to hVEGF even though 10 times the amount of hVEGF was used for hAng-1. In each case, the cell proliferation increases over the two week incubation period. This is due to the fact that there is more protein amount present in the NPs supernatant as days increase. The highest cell proliferation was observed for NP supernatant containing both hAng-1 and hVEGF which

was 89.62%, using NP supernatant containing hVEGF (Ang-1 antibody added) the proliferation was 67.79%, and using NP supernatant containing hAng-1(VEGF antibody added) the proliferation was 5.87%. This shows that using both hAng-1 and hVEGF together will produce the best results.

4.5.5 Retention of antiapoptotic activity of released hAng-1 and hVEGF on HUVECs

Serum deprivation method was used to induce apoptosis in the HUVECs to demonstrate the antiapoptotic effect of the both the proteins hAng-1 and hVEGF released from the NPs. Under serum deprivation, the HUVECs seeded in the 96 well plate undergo apoptosis and get detached from the wells. As demonstrated by Kwak et al, hAng-1 acts as an apoptotic survival factor for endothelial cells [121]. It was also shown that even when the inhibitory doses of hAng-1 was ten times more than hVEGF, both caused identical antiapoptotic effects in HUVECs. In addition, it was also observed that during serum starvation of HUVECs, the antiapoptotic effect of hAng-1 was significantly increased when hVEGF was added leading to a combined apoptotic inhibitory effect.

In our study, hAng-1 and hVEGF were loaded into NPs and the combined antiapoptotic effect of the proteins on the seeded HUVECs was demonstrated. It has been highlighted already that due to their very limited in vivo half-lives, the proteins have to be administered through multiple injections so as to achieve the desired therapeutic effect. Moreover, these macromolecules have high molecular weight which leads to low bioavailability, have high aqueous solubility and are incapable of diffusing through biological membranes [128, 129]. The use of NPs for the delivery of these macromolecules, which are in fact very unstable, could aid in protecting them from degradation in the biological environment of the organism. The HUVECs were then seeded and 0.1mL of NP supernatant from day 2, 8 and 14. NP supernatant containing BSA and serum free media without addition of hVEGF and hAng-1 taken as controls were added to the well and incubated for 96 hours. As shown in the **(Figure 4-6)**, the HUVECs with serum free control media and NP supernatant containing BSA always showed a percent apoptosis above 90%. The release kinetics shows that there is an increase in the cumulative amount of proteins released from the NPs. In the study, 0.4mL solution was taken from NPs supernatant and then replaced with serum-free media on the indicated days. The amount taken also has increasing protein content as the days increase. Due to this increasing protein presence, the percent cell apoptosis

decreases from day 2 to 14. The percent apoptosis due to NP supernatant containing hAng-1 (hVEGF antibody added) reduced from 31.5% to 27.06% for day 2 to day 14. Similarly, percent apoptosis due to NP supernatant containing hVEGF (hAng-1 antibody added) reduced from 29.6% to 27.74% for day 2 to 14. The combined antiapoptotic effect of the NP supernatant containing hAng-1 and hVEGF decreased from 16.44% to 10.42% for day 2 to 14. Thus, in comparison to control, the cell apoptosis decreased dramatically (almost by 50%) with the application of supernatant from NPs loaded with both the proteins.

(Figure 4-7) illustrates the antiapoptotic effect of hAng-1 and hVEGF on HUVECs for day 14. It was observed that on day 14 the proteins coencapsulated in the NPs significantly decreased HUVEC apotosis compared to the control as well as the individual proteins, The HUVECs were seeded onto a 96-well plate at a cell density of $2x10^4$ cells per well. After 24hours, the medium was then replaced with 0.1mL of NP supernatant, NP supernatant with excess hVEGF antibody, NP supernatant with excess hAng-1 antibody and incubated for 96 hours as shown in **(Figure 4-7A, B, C)**. Control cultures received the same amount of serum-free media without proteins as shown in **(Figure 4-7D)**.

4.6 Conclusion

In summary, we examined the *in vitro* effect of application of the angiogenic proteins, hVEGF and hAng-1, coencapsulated in albumin NPs towards therapeutic angiogenesis. Our results demonstrate that application of a NP system provided slow, continuous and extended release kinetics of the proteins which in turn resulted in an augmented combined effect of the released angiogenic proteins towards proliferation and anti-apoptosis of HUVECs in comparison to the released individual proteins. Future perspectives could involve *in vivo* studies demonstrating the potential of clinical application of this system towards therapeutic angiogenesis.

4.7 Acknowledgments

This work is supported by research grant to S.P. from Canadian Institute of Health Research (MOP 64308). A.P. acknowledges the financial support from Natural Sciences and Engineering Research Council (NSERC, Canada) - Alexander Graham Bell Canada Graduate Scholarship**.** S.A. is supported by the McGill Faculty of Medicine Internal Studentship - G. G. Harris

Fellowship. The authors thank Dr. Xue-Dong Liu of McGill University, Physics Department for the TEM imaging.

4.8 List of Figures and Tables

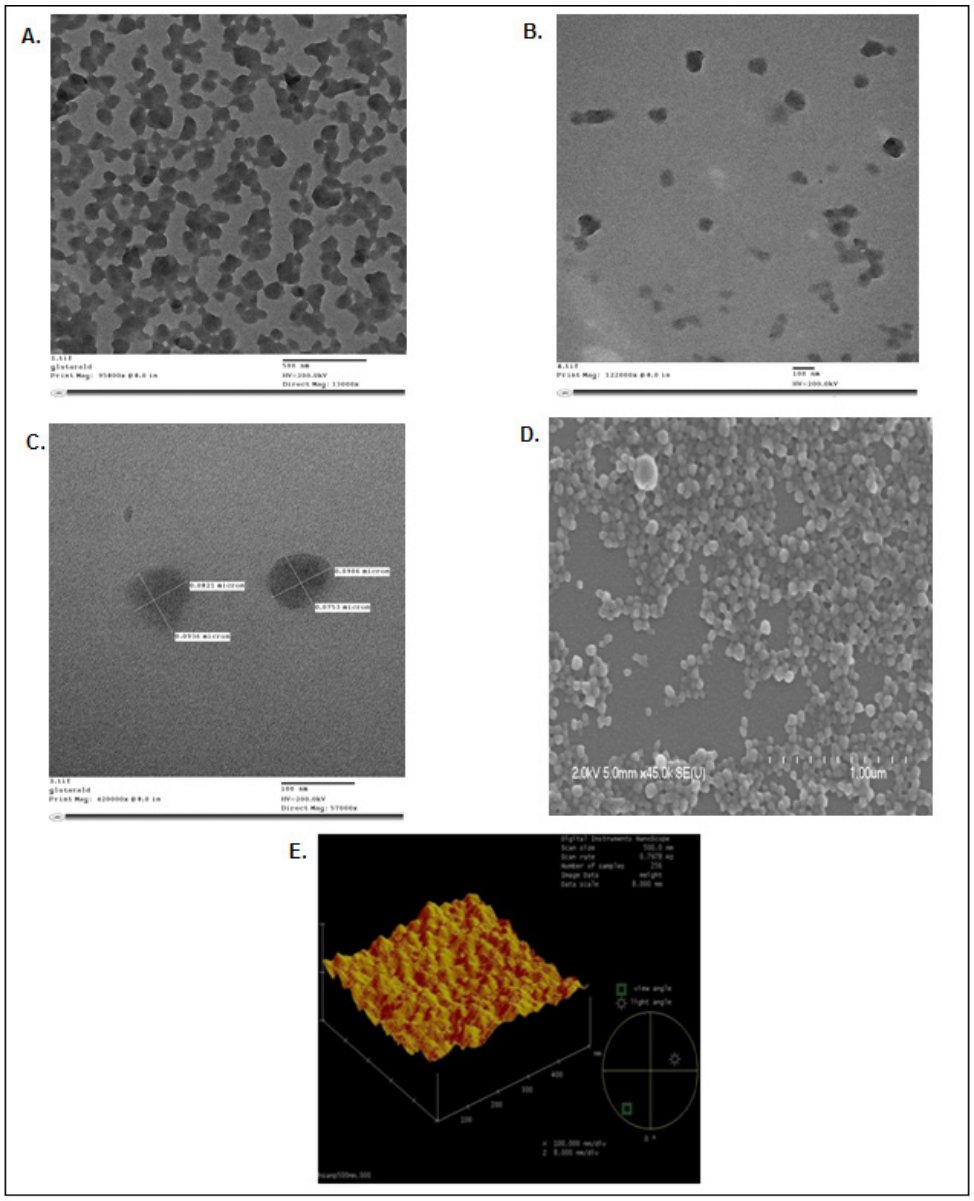

Figure 4-1: The TEM, SEM and AFM micrographs of the NPs illustrate that the particles are properly dispersed, spherically shaped and have smooth surface characteristics.
(A, B, C) TEM images for the NPs cross-linked with 40µl of 5% (w/v) glutaraldehyde, 200 kV resolution. **(A)** TEM image of the NPs before ultrasonication which indicates that before ultrasonication the NPs form clusters and are not properly dispersed. Bar, 500 nm **(B)** TEM image of the NPs after ultrasonication for 15 minutes. Here it is demonstrated how ultrasonication assists in separating out the particles more uniformly and the particles are properly dispersed. Bar, 100 nm **(C)** TEM image of the NPs after magnification. Bar, 100 nm **(D)** SEM image for the NPs cross-linked with 40µl of 5% (w/v) glutaraldehyde which demonstrates the smooth surface characteristics of the NPs, 2 kV resolution. Bar, 1 µm **(E)** Three-dimensional AFM image of the NPs. Bar, 300 nm.

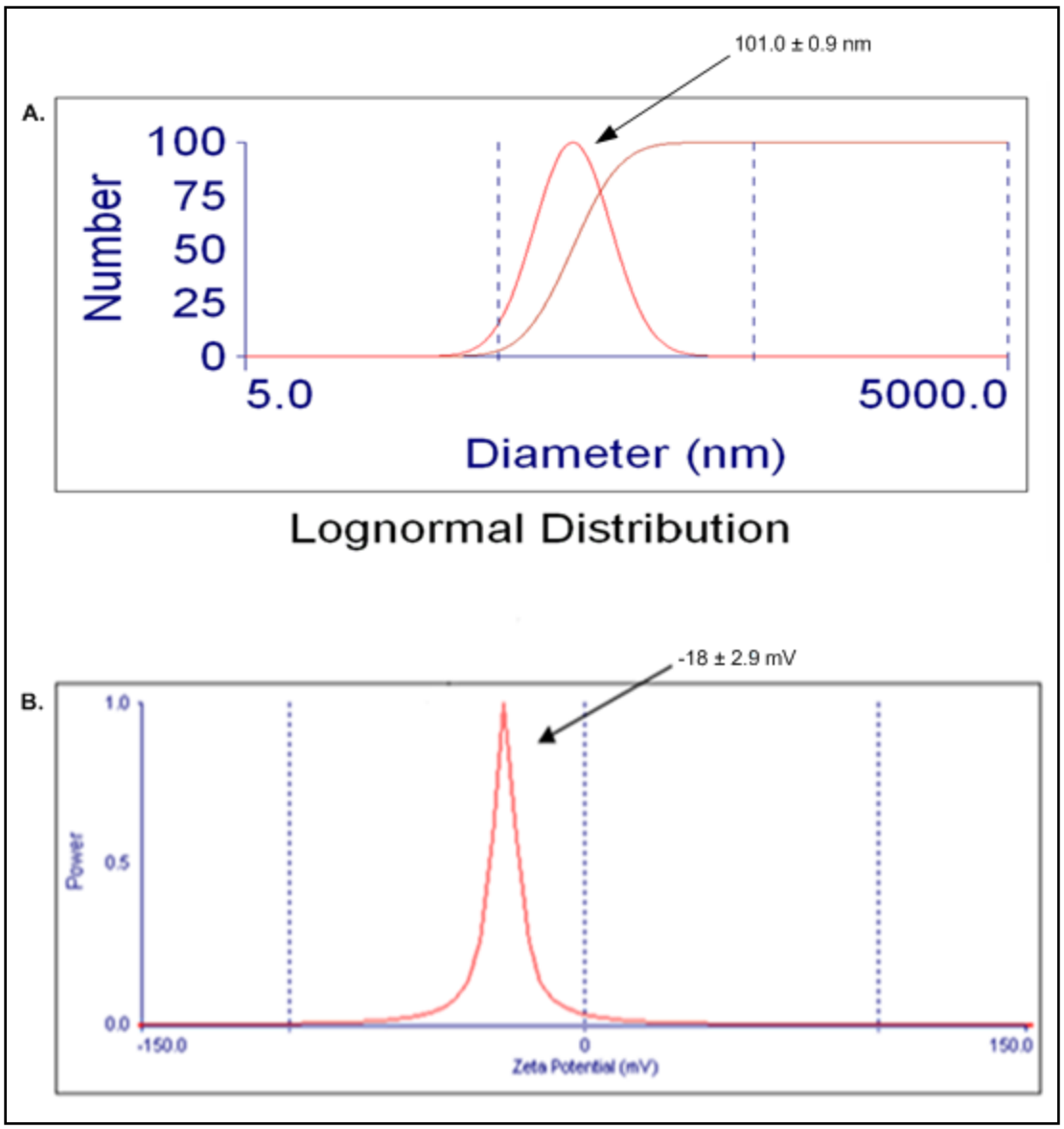

Figure 4-2: Zeta potential and particle sizer.

(A) The NPs were prepared by adjusting the pH of the HSA solution to 8.0 and controlling the amount of coacervation agent to form NPs of size approximately 101.0± **0. 9** nm **(B)** The protein NPs were negatively charged and the zeta potential was -18± **2. 9** mV at pH 7.0. The zeta potential of the glutaraldehyde cross-linked NPs decreases when the concentration of glutaraldehyde is increased. Zeta potential measurements were performed in phosphate buffer at pH 7.0 as pH and ionic strength of the dilution medium affects the magnitude of the zeta potential. The polydispersity index was found to be 0.3.

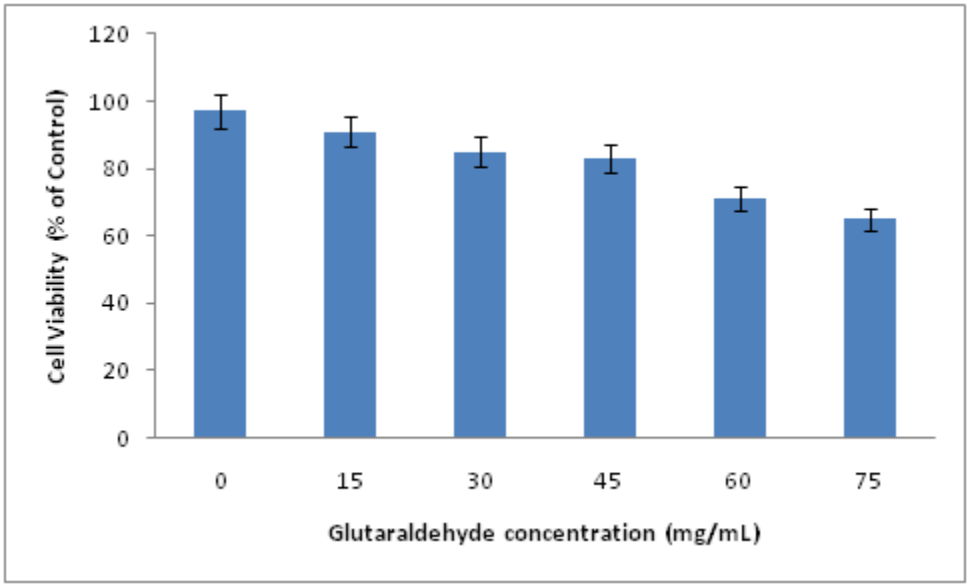

Figure 4-3: The cytotoxicity of the blank NPs cross-linked with a series of glutaraldehyde concentrations was studied by incubating HUVECs for 96 hours with the NPs.
Percent cell viability over initial number of HUVECs treated with NPs for up to 96 hours is shown. MTS assay was used to determine the cell viability after exposure to the NPs. NPs without glutaraldehyde coating showed a cell viability of above 90% after 96 hours. Increasing the amount of glutaraldehyde in the particle preparation increased the cytotoxicity of the NPs to the seeded HUVECs.

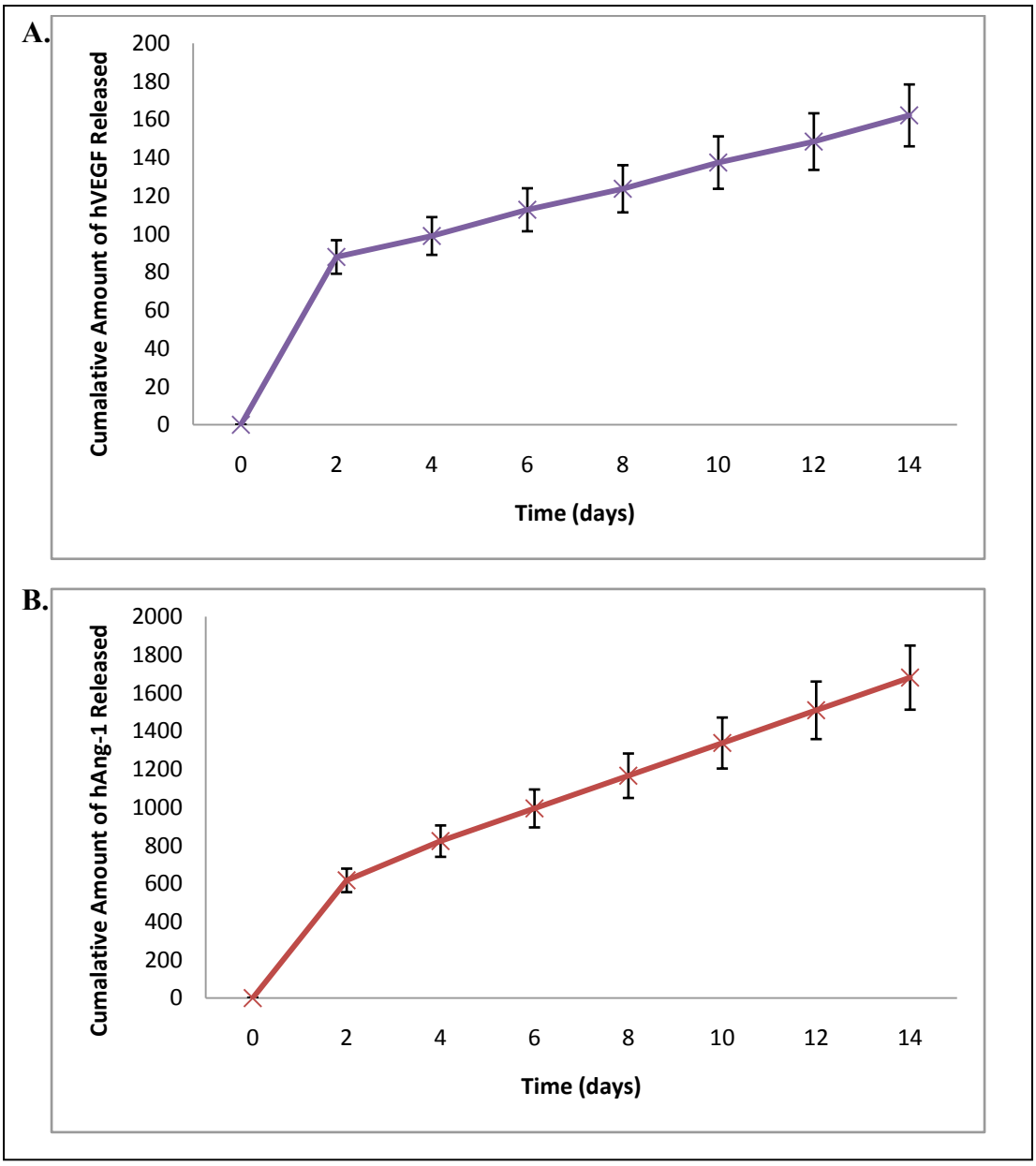

Figure 4-4: NPs sustain in-vitro protein release.

A and **B** Indicate the cumulative amount of hAng-1 and hVEGF released from the NPs over a time period of two weeks. At the end of the two week incubation period it was observed that 49% of hAng-1 and 59% of hVEGF had been released from the NPs.

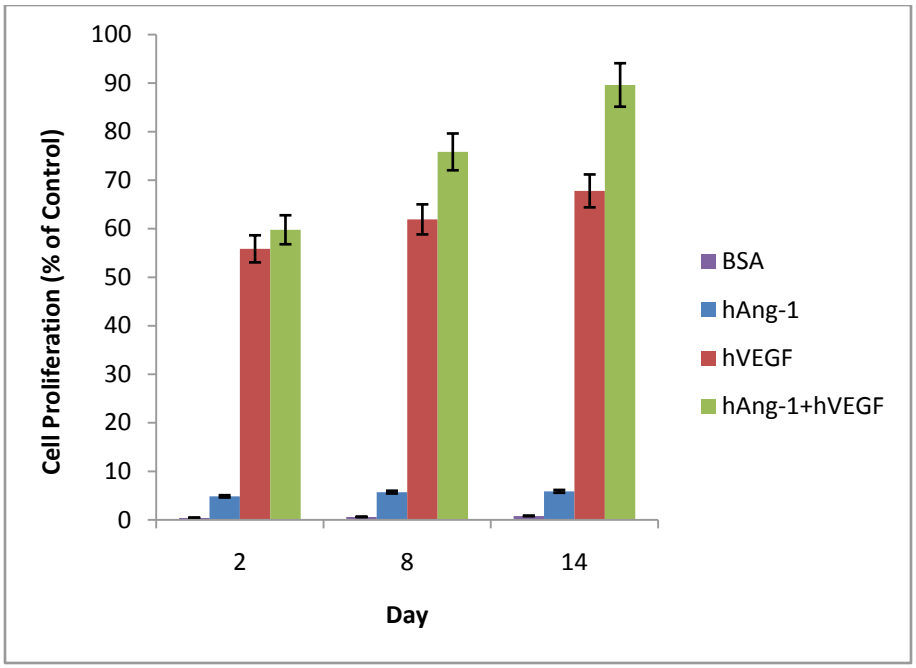

Figure 4-5: Bioactivity of hAng-1 and hVEGF loaded in NPs.

The proliferation of HUVECs in response to the NP supernatant was observed. HUVECs were seeded in a 96 well plate and the ECM was replaced with NP supernatant with hVEGF antibody, NP supernatant with hAng-1 antibody NP supernatant containing both hAng-1 and hVEGF, NP supernatant containing BSA and ECM (with 5% FBS) as the controls for 96 hours. The results were illustrated as the percent increase in cell proliferation relative to the unstimulated control. Least cell proliferation is observed with hAng-1 and maximum proliferation is observed in supernatant with a combination of both hAng-1 and hVEGF. The supernatant with BSA showed negligible effect as expected. An increasing cell proliferation is observed over the two week incubation period.

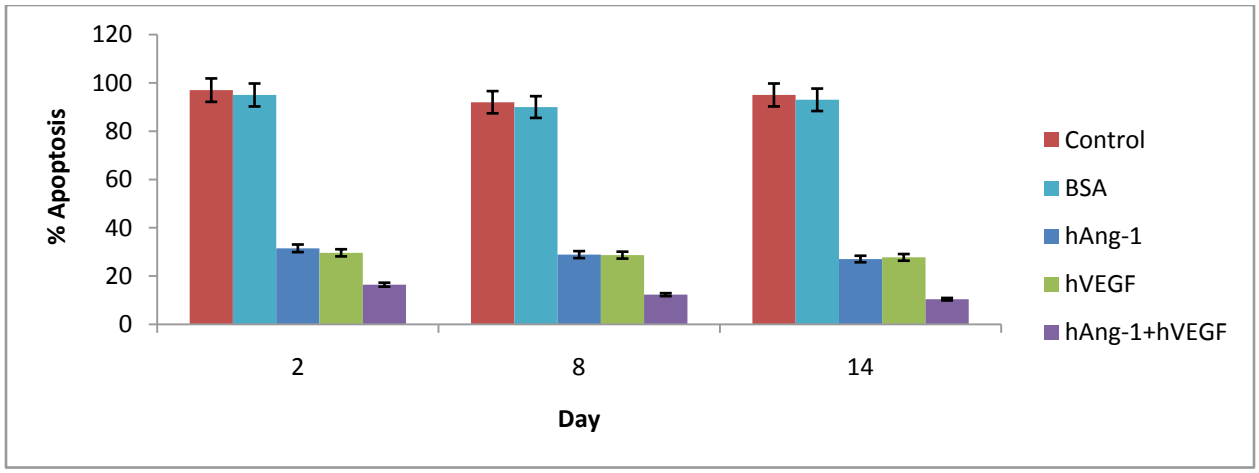

Figure 4-6: hAng-1 and hVEGF were loaded into NPs and the combined antiapoptotic effect of the proteins on the seeded HUVECs was demonstrated.

The HUVECs treated with serum free control media without addition of hVEGF and hAng-1 proteins, always showed a percent apoptosis above 90%. The release kinetics shows that there is an increase in the cumulative amount of proteins released from the NPs. The percent apoptosis due to NP supernatant containing hAng-1 (hVEGF antibody added) reduced from 31.5% to 27.06% for day 2 to day 14. Similarly, percent apoptosis due to NP supernatant containing hVEGF (hAng-1 antibody added) reduced from 29.6% to 27.74% for day 2 to 14. The combined antiapoptotic effect of the NP supernatant containing hAng-1 and hVEGF decreased from 16.44% to 10.42% for day 2 to 14. Thus, in comparison to control, the cell apoptosis decreased dramatically (almost by 50%) with the application supernatant from NPs loaded with both the proteins.

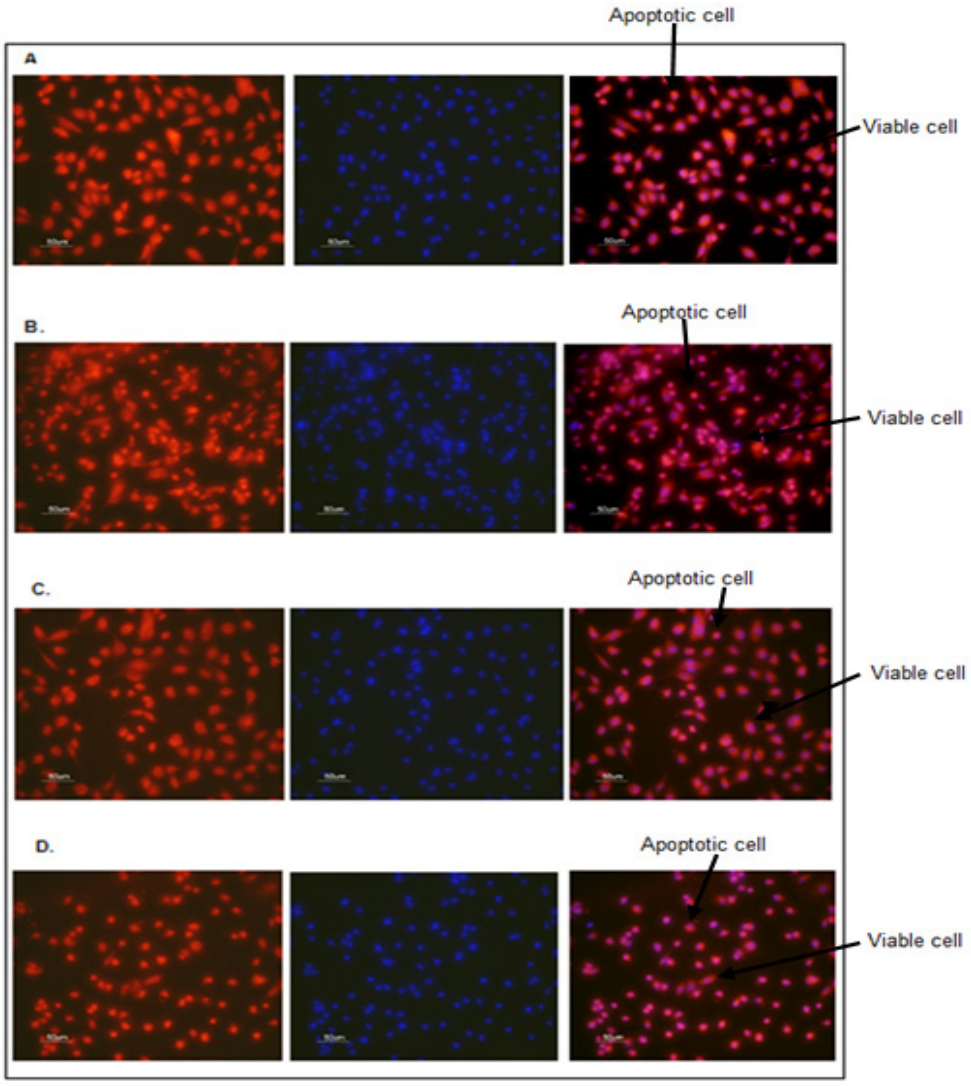

Figure 4-7: Fluorescent microscope images showing antiapoptotic effect of hAng-1 and hVEGF on HUVECs for day 14.

Bar, 50 µm It was observed that on day 14 the proteins coencapsulated in the NPs significantly decreased HUVEC apoptosis compared to the control as well as the individual proteins, HUVECs were seeded onto a 96-well plate at a cell density of $2x10^4$ cells per well. The medium was then replaced with 0.1mL of **(A)** NP supernatant (**B)** NP supernatant with excess hVEGF antibody **(C)** NP supernatant with excess hAng-1 antibody and incubated for 96 hours. **(D)** Control cultures received the same amount of serum-free media without addition of hVEGF and hAng-1 proteins. The numbers of cells floating in each well were collected after PBS washing and counted. The number of apoptotic cells in the adherent cells was then determined after 96 hours using MitoTracker® Red CMXRos kit and DAPI nucleic acid staining and counted using fluorescence microscope.

5 Controlled delivery of bone marrow stem cell-derived growth factors using polymeric nanoparticles improves myocardial repair and function in infarcted rat heart model

Research article

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5.1 Preface

In chapter 4, the mitotic and anti-apoptotic effects of using a combination of key pro-angiogenic proteins, hVEGF and hAng-1, co-encapsulated in HSA NPs on HUVECs were studied.In this study, we move towards the main goal of the thesis where stem cell growth factor cocktail harnessed from bone marrow derived stem cells was encapsulated into PLGA NPs for a more controlled and protected delivery. Particle characterization studies of the NPs were first performed followed by a three week *in vitro* release study of a key pro-angiogenic protein, hVEGF, present in the factor cockail released from the PLGA NPs. Biofunctional activity of hVEGF present in the released factor cocktail was also assessed through *in vitro* angiogenic assays using endothelial cells. Next, pre-clinical studies were done to analyze the therapeutic effect and benefit of using PLGA NPs loaded with the stem cell cocktail for potential therapeutic application in inducing cardiac tissue regeneration using a rat model of myocardial infarction. We intramyocardically delivered the factor cocktail loaded PLGA NPs and compared the effect to control empty PLGA NPs and free CM.

5.2 Abstract

The use of MSCs for potential application in cardiac tissue regenerative therapy following myocardial infarction (MI) has been researched in-depth over the past years. However, this stem-cell mediated improvement in tissue remodeling has recently been attributed to stem cell-derived growth factors as being the key players and evidence supporting this hypothesis is on the rise. Although the injection of these stem cell harnessed factors has shown success, we propose that the use of a biocompatible delivery vehicle allowing sustained and controlled release of the encapsulated stem cell GFs in addition to protecting them from the harsh *in vivo* environment, which could augment the efficiency of this stem cell paracrine effect and in fact completely eliminate the concept of stem cell administration into the heart. With this goal, in this study we developed PLGA nanoparticles (NPs) loaded with concentrated biologically active GF cocktail secreted by MSCs to mimic the stem cell paracrine effect to induce myocardial angiogenesis following MI. *In vitro* studies were performed to observe the release of hVEGF present in the stem cell secreted GFs or conditioned medium (CM) from the NPs followed by bioactivity assessment of the released CM. During *in vivo* evaluation, we intramyocardically delivered the PLGA NPs loaded with the concentrated CM to an acute MI rat model and compared the effect to control empty PLGA NPs and free CM. We observed that the PLGA NP group loaded with GFs resulted in an increased capillary and arteriole density with a decreased infarct size and a significantly greater LV wall thickness in contrast to the other two groups. Furthermore, echocardiography data demonstrated that cardiac function was significantly higher in the CM loaded PLGA NP group compared to the control and free CM group.

Keywords: Stem cells, growth factors, nanoparticles, angiogenesis, controlled release, myocardial infarction

5.3 Introduction

The onset of myocardial infarction results in the irreversible loss of cardiomyocytes, which in turn leads to an impaired ventricular function [143]. Several clinical trials demonstrate that stem cell regenerative therapy could be a potential approach to repopulate the injured myocardium, through the intramyocardial transplantation of adult stem cells and hence improve functionality of the damaged myocardial tissue [5]. Studies involving the administration of bone marrow derived stem cells (BMSCs) have demonstrated their potential in promoting the repair of the

damaged heart tissue by inducing therapeutic angiogenesis and revascularization [5, 6]. Although intense research has been focused on the understanding the regenerative ability of these transplanted stem cells, controversy over the mechanisms underlying their therapeutic effects has arisen. Current studies now provide evidence for the possibility of a paracrine effect, created by the secretion of growth factors from the administered stem cells, as the key mediator in cardiac protection and repair following MI [8, 9]. It has been demonstrated that the intramyocardial administration of BMSCs in *in vivo* MI rat and mice models significantly augmented heart repair and function as early as 3 days which provided further evidence of the possibility of paracrine mechanisms responsible for such early recovery instead of cellular diffusion or fusion [144]. Recent studies have shown that BMSCs under hypoxic or serum starvation conditions secrete augmented amounts of a wide range of pro- angiogenic growth factors or proteins into the BMSC-conditioned medium (BMSCCM) such as hVEGF [8, 10]. Injection of these stem cell-derived growth factors generated under hypoxic conditions directly into small as well as large animal MI models has shown to significantly augment cardiac regeneration and ventricular function [54, 145, 146]. Although the use of stem cells for cellular therapy has been researched in depth, studies involving the possible application of stem cell-derived growth factors could overcome the administration of stem cells and the drawbacks related to their use such as poor retention in the myocardium after injection and generation of a local immune response [14]. Other researchers have also studied the effect of administration of individual or dual natural recombinant human proteins to promote neovascularization of the damaged tissue supported with MI models [42, 59]. However, the use of a cocktail of stem cell-derived growth factors is hypothesized to have an augmented effect in comparison to administration of just single or dual proteins.

With emerging evidence supporting the paracrine hypothesis the need for an efficient delivery system aimed at enhancing the therapeutic effectiveness of the administered stem cell CM becomes evident. Although initial results with pro-angiogenic proteins directly injected as a bolus have been promising, the effect is short lived due to protein instability or denaturation after administration [16, 17]. The use of a delivery vehicle for the hBMSC CM would not only protect the protein cocktail from the harsh *in vivo* environmental conditions but would also enable a more sustained and controlled release of the growth factors at the target site thus mimicking the endogenous release profiles of proteins during tissue regeneration. Poly (D, L-lactide-co-

glycolide) (PLGA) copolymer due to its attractive properties of excellent biocompatibility, biodegradability and sustained release has been approved by the Federal Drug Administration and has been widely used in several drug delivery applications [91]. Furthermore, PLGA MPs have been used in numerous studies for delivery of bioactive substances, supported with promising cardiac tissue regeneration and remodeling results shown in *in vivo* animal models [18, 93]. However, the focus of current studies is now slowly moving towards the possible application of PLGA NPs instead of MPs as a delivery vehicles due to several advantages such as the lesser risk of embolization and a larger surface area [74, 147].

In our study, we propose the use of biocompatible PLGA NPs as delivery systems for hBMSC-CM allowing a more controlled and extended therapeutic effect. *In vitro* studies using the hBMSC-CM PLGA NPs were done to observe the release profile of the pro-angiogenic protein, hVEGF present in augmented amounts in the CM when generated from the stem cells under hypoxic conditions, followed by assessment of the functional activity of the released hBMSC CM through *in vitro* angiogenic assays for Human umbilical vein endothelial cell (HUVEC) proliferation, migration and tube formation. Next, a rat model of MI was used to analyze the effect and efficiency of the stem cell-derived growth factors loaded the PLGA NP system for potential therapeutic application in inducing cardiac tissue regeneration after MI. The entire procedure of stem cell-derived growth factor loaded PLGA NP preparation followed by *in vivo* analysis is illustrated **(Figure 5-1)**.

5.4 Materials and Methods

5.4.1 Cell culture

Human mesenchymal bone marrow stem cells (hBMSC) and Human umbilical vein endothelial cells (HUVEC) were purchased from Sciencell (Carlsbad, California, USA) and were cultured and expanded in 75cm^2 cell culture flasks (Corning, NY, USA) at 37^{o}C and under 5% CO_2 atmosphere. Both cell lines were grown in culture medium supplemented with fetal bovine serum (FBS, 5% (v/v)) and cell growth supplements with penicillin/streptomycin solution from the same suppliers, where the hBMSCs were cultured in Mesenchymal Stem Cell Medium (MSCM) and HUVECs were grown in Endothelial cell medium (ECM) according to instructions. The cells in passages 4 and 5 were used in the experiments.

5.4.2 Generation of the CM from hBMSCs

After the hBMSCs had reached a confluency of approximately 90-95%, the MSCM with 5% FBS was removed and the cells were then cultured under hypoxic conditions (5% CO_2 and balanced N_2) with serum free MSCM in a Billups-Rotenberg modular incubator chamber. The incubator is flushed for 5-10 minutes at 10-15 l/min and placed in the 37^{o}C incubator for 16 hrs. At the end of the incubation period, the hBMSC conditioned medium (hBMSC CM) generated was then collected and filtered through a 0.22 μm syringe filter. The growth factors were concentrated using Millipore Amicon centrifuge filters (Millipore, Bedford, MA, USA).

5.4.3 Formulation of hBMSC CM loaded PLGA NPs

The hBMSC CM loaded PLGA NPs were prepared by a modified double emulsion-solvent evaporation technique as previously described [148]. Briefly, the concentrated hBMSC CM was added to 50 mg Poly (D, L-lactide-co-glycolide) (PLGA, RESOMER RG 504H, Sigma-Aldrich, USA) dissolved in 1 ml dicloromethane (DCM, ACP Chemical Inc., Montreal, Quebec). Albumin from bovine serum (BSA, Sigma-Aldrich, USA) was also added to the solution to maintain stability of the growth factors during encapsulation procedure as done previously. The primary emulsion was generated by a high speed homogenizer operating for 2 mins at 3000 rpm. The double emulsion was obtained by adding the emulsion to 10 mL of 1.5% (w/v) Poly (vinyl alcohol) (PVA, Sigma-Aldrich, USA) and followed by homogenizing for 2 mins again after incubation on ice. The resultant emulsion was then placed under magnetic stirring for 3 hrs at room temperature so as to remove the organic solvent. After 3hrs, the hardened PLGA NPs

loaded with the hBMSC CM were collected by centrifugation at 10,000 x g and then washed three times with distilled water. The NPs were prepared in three different batches and lyophilized at -20^{o}C for further experiments. As negative control, empty PLGA NPs without CM were also prepared.

5.4.4 Nanoparticle shape, surface morphology and size

The external shape and morphology of the loaded PLGA NPs was analyzed using Scanning Electron Microscopy (SEM). The NPs were first dispersed in ddH_2O and then dried under room temperature using natural convection. The samples were then examined under S4700 FEG-SEM (Hitachi, Oakville, Ontario, Canada). Transmission Electron Microscopy (TEM) was used for size characterization by suspending the PLGA NPs in 1X PBS and observed using a CM200 FEG-TEM (Philips, Markham, Ontario, Canada).

5.4.5 Loading efficiency of hVEGF present in hBMSC CM within the NPs

In order to determine the encapsulation efficiency of hVEGF protein present in the hBMSC CM loaded within the NPs, the prepared hBMSC CM NPs were completely degraded by adding 1ml of 0.5N NaOH and placed under constant stirring for 24hrs as previously performed [93]. The solution was then centrifuged and the amount of hVEGF was analyzed using hVEGF ELISA assay as per manufacturer's instructions. Data was collected from three independent experiments and the results were expressed as loading efficiency (ratio of actual to theoretical loading x 100) ± standard deviation.

5.4.6 In vitro release kinetics of hVEGF present in the hBMSC CM from PLGA NPs

To determine the in vitro release of hBMSC CM from PLGA NPs, the NPs were suspended into 10 ml of serum free ECM with 1% penicillin/streptomycin at 37°C under constant shaking. At scheduled time intervals, 0.2 ml of the released medium was withdrawn and then replaced with fresh ECM. To detect the concentration of hVEGF present in the hBMSC CM released into the medium, the samples were first centrifuged at 20,000 x g for 10 min at 4^{o}C and the supernatant was analyzed using hVEGF ELISA assay according to manufacturer's instructions. Data was collected from three independent experiments and represented as cumulative nanogram (ng) of hVEGF released (mean ± standard deviation of three replicates) versus day of release.

5.4.7 HUVEC proliferation assay

The proliferative capacity of HUVECs when incubated with the released hBMSC CM from loaded PLGA NPs was used to determine the bioactivity of hVEGF present in the CM. HUVECs from passages three to five were used for all the experiments. The CellTiter 96® AQueous Non-Radioactive Cell Proliferation MTS Assay kit was used to evaluate the proliferation of the HUVECs. The HUVECs (2 x 10^4cells/well) were seeded in a 96 well plate and incubated for 24 hrs with ECM supplemented with 5% (v/v) FBS and cell growth supplements. The ECM was then removed and the cells were washed with PBS and were incubated with 0.2 ml of the 50% fresh ECM supplemented with 5% (v/v) FBS without cell growth supplements and 50% of the supernatant from PLGA NPs loaded with CM (incubated with 10 ml serum free ECM) from day 4, and CM released from the PLGA NPs for day 4 with 10µg/mL anti-hVEGF antibody. Control wells received 0.2 ml of the 50% fresh ECM supplemented with 5% (v/v) FBS without cell growth supplements and 50% of supernatant when the empty PLGA NPs were placed in 10 ml serum free ECM. Briefly, after 96 hours of incubation, data is represented as the mean percentage of endothelial cells on day 4 versus initial cell number (2 x 10^4cells/well) (± Standard deviation), determined by MTS assay performed following manufacturer's instructions. 20µl of MTS solution was added to each well and the HUVECS were incubated for 4hrs in 37^{o}C. The absorbance was measured at 490 nm using the 1420-040 Victor3™ Multilabel Counter (Perkin Elmer, Woodbridge, Ontario, Canada). The concentration of hVEGF in the hBMSC CM released into the supernatant was determined using hVEGF ELISA assay.

5.4.8 HUVEC Migration Assay

The ability of the supernatant containing hBMSC CM released from the PLGA NPs to induce HUVEC migration was also evaluated using the Oris® cell migration assembly kit-flex (Platypus Technologies, Madison, WI) according to manufacturer's instructions. HUVECs were seeded at 25,000 cells/well in a 96 well plate fitted with stoppers with ECM (5% FBS with cell growth supplements) and allowed to adhere overnight to the plates. Then, once the cell monolayer had reached confluency, the cell seeding stoppers were removed from the wells and cells were suspended with 0.2 ml of the 50% fresh ECM supplemented with 5% (v/v) FBS without cell growth supplements and 50% of the supernatant from PLGA NPs loaded with CM (incubated with 10 ml serum free ECM) from day 4, and CM released from the PLGA NPs on day 4 with 10µg/mL anti-hVEGF antibody. Control wells received 0.2 ml of the 50% fresh ECM

supplemented with 5% (v/v) FBS without cell growth supplements and 50% of supernatant when the empty PLGA NPs were placed in 10 ml serum free ECM. Following HUVEC migration, the cells were stained with Calcein AM (Invitrogen) and the cell migration was assessed under 10X magnification using a fluorescence microscope. The number of cells which had moved across the starting line (mean ± Standard deviation of three independent experiments) was assessed using image J software.

5.4.9 HUVEC Tube formation Assay

The ability of the released CM from PLGA NPs to induce HUVEC tube formation was assessed using the endothelial tube formation assay (Cell Biolabs, Inc, San Diego, USA) according to manufacturer's protocol. 50µL of ECM gel was added to a 96-well plate and incubated for one hr at 37°C to enable gel formation. HUVECs (2×10^4cells/well) were seeded in a 96 well plate and incubated for 24 hrs with ECM supplemented with 5% (v/v) FBS and cell growth supplements. The ECM was then removed and the cells were washed with PBS and were incubated with 0.2 ml of the 50% fresh ECM supplemented with 5% (v/v) FBS without cell growth supplements and 50% of the supernatant from PLGA NPs loaded with CM (incubated with 10 ml serum free ECM) from day 4, and CM released from the PLGA NPs on day 4 with 10µg/mL anti-hVEGF antibody. Control wells received 0.2 ml of the 50% fresh ECM supplemented with 5% (v/v) FBS without cell growth supplements and 50% of supernatant when the empty PLGA NPs were placed in 10 ml serum free ECM. The plate was incubated at 37°C for 8-12 hrs to allow the cell adhesion to the gel coating. After incubation period, the medium was removed and the cells were then incubated with 50µL of 1X Calcein AM for 30 mins at 37^{o}C. The cells were washed twice with 1X PBS and the endothelial capillary-like tube formation in each well was examined using a fluorescent microscope under 10X magnification and the network was analyzed by Image J software. The results were quantified as the mean tube length ± Standard Deviation in µm of the HUVEC-made capillary network as shown previously [149]. Administration of CM loaded PLGA NPs in MI rat model.The *in vivo* myocardial infarction model used in our study comprised of immunocompetent female Lewis rats (200 to 250 gm, Charles River, QC) used according to previously established protocol [150, 151]. The procedures used were followed in compliance with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23) and the Guide to the Care and Use of Experimental Animals of the Canadian Council on Animal Care. 5% isoflurane was used to anesthetize the rats in an induction chamber followed by intubation

using an 18-gauge catheter and mechanical ventilation (Harvard Ventilator, PQ, Canada) at 80 breaths/min (3% isoflurane was maintained for continuous anesthesia throughout the procedure). A left thoracotomy through the fourth intercostal space was carried out to expose the left ventricle and the left coronary artery was ligated with a 7-0 polypropylene suture (Ethicon, Inc, Somerville, NJ) 2 mm from its origin. After ligation of the coronary artery, the rats were subjected to 3 equal 100 µl left ventricular direct intramyocardial injections using a 27 gauge needle at the peri-infarct regions. The same volume of PBS (300µl) was used as the delivery agent for all 3 of the experimental groups. The PLGA NP $_{CM}$ group (n=6) received PLGA NPs encapsulating concentrated CM containing 400ng of hVEGF and the control group (n=6) received an equivalent amount of empty PLGA NP suspension. The positive control group with Free CM (n=6) received free concentrated CM containing 100ng of hVEGF. The chest was closed at the end of the procedure and the rats were allowed to recover.

5.4.10 Histological analysis for scar area detection

4 weeks post-operation, rats were anesthetized and sacrificed by rapid excision of the heart for subsequent histological analysis. To the remove excess blood from the ventricles, the excised hearts were immediately soaked in cold saline. The hearts were fixed in neutral-buffered 4% formalin and then the paraffin embedded samples were sectioned at 5µm. In order to delineate the scar tissue (blue color) from the total area of myocardium, Masson's trichrome staining (*DBS,* Pleasanton, CA) was performed according to manufacturer's instructions and the stained sections obtained were captured as digital images and analyzed using Image J-1.41 software [152]. The infarct size and wall thickness in the left ventricles at the section of the middle point between ligation and apex were measured as previously described [153]. Infarct area and ventricular wall thickness were calculated and expressed as a percentage.

5.4.11 Immunohistochemistry for detecting neovascularization

To quantify the extent of neovascularization after treatment, the capillary and arteriole density in the peri-infarct area were analyzed according to previous studies [152]. For detection of the capillary density, using 3 tissue sections spanning the peri-infarct tissue region of each animal, staining was performed with anti-PECAM (Santa Cruz) and the endothelial cells were identified. After imaging the sample slides under 20X magnification, the capillary density (mean total PECAM-positive microvessels)/mm^2 was quantified in four

fields in the peri-infarct area. For arteriole density detection, anti-smooth muscle α-actin (Santa Cruz) was used to identify the smooth muscle cells. Similarly, arteriole density was also quantified as above as (mean total smooth muscle α-actin-positive microvessels)/mm^2.

5.4.12 Echocardiogram analysis

Echocardiography was performed using a commercially available system (SonoSite, Titan-Washington, Seattle, WA) equipped with a 15-MHz transducer for all the rat groups: group 1 with PLGA NP $_{Empty}$ (n=6), group 2 with free CM (n=6) and group 3 with PLGA NP $_{CM}$ (n=6) on day 3 and day 28 post infarction as done in previous studies [150, 151].

5.4.13 Statistical Analysis

Data are presented as mean ± Standard Deviation (SD) from independent experiments as described in the figure legends. Statistics were performed using two-way and/or one-way ANOVA by Bonferroni's multiple comparison post-hoc test. All statistical analyses were performed with Prism 5 (GraphPad Software).

5.5 Results

5.5.1 Characterization of hVEGF amount in CM, PLGA NP shape, surface morphology, size, loading efficiency and release kinetics

The quantitative amount of hVEGF (53.5 ± 15.5 ng/10^6 cells) in the hBMSC CM secreted from the hBMSCs after incubation for 16 hrs with serum free MSCM under hypoxic conditions was determined using hVEGF ELISA assay.

SEM and TEM images **(Figure 5-2A, B, C, D)** showed homogenous hBMSC CM loaded PLGA NPs, perfectly spherical in shape with a smooth surface devoid of cracks. The PLGA NPs so formed using double emulsion-solvent evaporation technique had an average nano-size ranging between 100 and 400 nm. To prevent protein deactivation, BSA was co-encapsulated with the hBMSC CM during the particle preparation.

The total amount of hVEGF present in hBMSC CM loaded within the PLGA NPs was measured by dissolving the particles in NaOH and the encapsulation efficiency was found to be (48.2 ± 2.5 %) as determined by the hVEGF ELISA assay. For both our *in vitro* and *in vivo* studies, the estimated dose of hVEGF in the hBMSC CM encapsulated in PLGA NPs was 8.01 ng per mg of polymer. Free hBMSC CM containing an equivalent of 100 ng of hVEGF was used as control.

The *in vitro* release profile of hVEGF in hBMSC CM from PLGA NPs was investigated at 37^{o}C for 21 days as illustrated **(Figure 5-2E)**. hVEGF release was characterized by two phases: an initial burst phase (25 ± 1.7 %) followed by a slower sustained release over time. The results also show that by day 21 of the release study, approximately (64.7 ± 3.7 %) of encapsulated hVEGF had been released from the NPs into the external medium.

5.5.2 Effect of hBMSC CM released from PLGA NPs on HUVEC proliferation

In our study, the CellTiter 96® AQueous Non-Radioactive Cell Proliferation MTS Assay kit was used to examine the *in vitro* ability of hBMSC CM released over time from the NPs to induce proliferation of human endothelial cells which is crucial for successful progression of angiogenesis [154]. Data is represented **(Figure 5-3)** as the mean percentage of endothelial cells on day 4 versus initial cell number (2 x 10^4cells/well) (± Standard deviation of 3 independent experiments) and it was observed that the HUVECs incubated with 50% ECM (5 % FBS without cell growth supplements) and 50% supernatant from loaded PLGA NPs containing CM (hVEGF amount 2.3 ± 0.5 ng) significantly enhanced the growth of HUVECs compared to wells receiving supernatant from empty NPs without CM. Supernatant from day 4 containing hBMSC CM released from the PLGA NPs resulted in a significant peak in HUVEC proliferation numbers in contrast to that from empty NPs (110.3 ± 5.34% vs 23 ± 3.01% of seeded HUVEC cells, $p<0.001$). The fact that supernatant from empty PLGA NPs did not significantly affect HUVEC proliferation however demonstrates that the NPs had no toxic effect on the cells. The individual stimulatory effect of hVEGF was demonstrated by the addition of anti-hVEGF antibody against the protein in the CM where the HUVEC cell number (61.7 ± 3.76%) significantly decreased in the presence of anti-hVEGF antibody. This inhibitory study however highlights that despite the presence of blocking antibody against hVEGF, there still remained a considerable increase in HUVEC number demonstrating the influence of the numerous cytokines present in the hBMSC CM released from the PLGA NPs. These results confirmed that the particle preparation process did not inversely affect the functional activity of the loaded stem cell-derived growth factor cocktail.

5.5.3 Effect of hBMSC CM released from PLGA NPs on HUVEC migration

The Oris® cell migration assembly kit-flex was used to examine the capacity of the CM released from the PLGA NPs on day 4 to induce HUVEC migration **(Figure 5-4B)**. It was observed that

after the stoppers were removed from the wells, HUVECs incubated with 50% ECM (5% FBS without cell growth supplements) and 50% supernatant with hBMSC CM released from the PLGA NPs (hVEGF amount 2.3 ± 0.5 ng) for 8-12 hrs migrated more as compared to the cells receiving unconditioned supernatant from empty NPs (61.33 ± 3.51 % vs 34.33 ± 4.5 %, $p<0.001$). Anti-hVEGF antibody was again added to the supernatant with released CM to also assess the extent of impact of hVEGF present in the hBMSC CM released from the NPs on HUVEC migration and as expected, a decrease in cell migration was observed (42.67 ± 3.5 %).

5.5.4 Effect of hBMSC CM released from PLGA NPs on HUVEC tube formation

The ability of the CM released from the PLGA NPs on day 4 to induce HUVEC tube formation was also studied. As illustrated in **(Figure 5-5A)** cells incubated with 50% ECM (5% FBS without cell growth supplements) and 50% supernatant with hBMSC CM released from the PLGA NPs (hVEGF amount 2.3 ± 0.5 ng) caused an enhanced effect on HUVEC capillary network formation as compared to the cells receiving unconditioned supernatant from empty NPs. The mean tube length was determined for both groups (273.3 ± 6.65 µm for PLGA NP $_{CM}$ vs 79 ± 5.56 µm for PLGA NP $_{Empty,}$ $p<0.001$) as shown **(Figure 5-5B)**. To also assess the extent of impact of hVEGF present in the hBMSC CM released from the NPs on HUVEC tube formation, anti-hVEGF antibody was added to the supernatant with released CM. A sharp drop in tube formation (155.3 ± 6.5 µm) was observed which provides evidence of the strong pro-angiogenic nature of hVEGF present in the CM. However, it also highlights the retained angiogenic functionality of the other growth factors in the CM which in still higher in comparison to supernatant from empty PLGA NPs. Released hBMSC CM significantly attenuates scar area of infarcted myocardium.

The extent of myocardial remodeling after infarction was observed via Masson's Trichome staining for all groups as illustrated **(Figure 5-6A)**. In all 3 groups, positively stained fibrous infarct areas in the heart 28 days post MI were clearly shown. Heart tissue sections in the PLGA NP $_{CM}$ treatment group had significantly lesser infarct areas than the other two groups **(Figure 5-6C)**, 18.46 ± 2.01 % for PLGA NP $_{CM}$; 32.15 ± 2.45 % for PLGA NP $_{Empty}$ and 21.9 ± 2.07 % for Free CM). Moreover, the animals in PLGA NP $_{CM}$ group even demonstrated a higher left ventricular wall thickness in comparison to the group administered with free cytokines **(Figure 5-6B)**, 1.85 ± 0.18 mm for PLGA NP $_{CM}$ vs. 1.49 ± 0.20 mm for Free CM) which further

provides evidence of the potential benefit to the delivery of the hBMSC CM via PLGA NPs in comparison to the administration of free CM. Here again, the animals treated with empty PLGA NPs showed the lowest left ventricular wall thickness (0.92 ± 0.11 mm).

5.5.5 hBMSC CM released from PLGA NPs induces angiogenesis and arteriogenesis

A total of 18 rats were used for *in vivo* analysis (Group1: PLGA NP $_{Empty,}$ n=6; Group 2: Free CM, n=6, Group 3: PLGA NP $_{CM}$, n= 6. To assess the neovasculature formation of the tissue in the peri-infarct area, the capillary **(Figure 5-7A)** and artery densities **(Figure 5-7B)** were measured. It was observed, as illustrated in **(Figure 5-7C)**, that the administration of hBMSC CM loaded PLGA NPs in group 3 animals did in fact lead to a significant improvement in angiogenesis in the compared to the injection of free cytokines (1070.42 ± 16.57 for PLGA NP $_{CM}$ vs 892.2 ± 19.92 for Free CM). The PLGA NP $_{Empty}$ group however showed a much lower capillary density (409.22 ± 15.82) compared to the other 2 groups. A similar augmented effect was observed towards promotion of arteriogenesis **(Figure 5-7D)**, where the group treated with PLGA NP $_{CM}$ showed a much higher arteriole density as compared to the group treated with Free CM (181.16 ± 11.14 for PLGA NP $_{CM}$ vs. 100.16 ± 10.22 for Free CM). Again compared to the other 2 groups, the animals administered PLGA NPs $_{Empty}$ did not show any significant improvement in arteriole density (58.2 ± 5.64) in infarcted heart.

5.5.6 hBMSC CM released from PLGA NPs significantly improves myocardial functional

The ejection fraction percentage in the rat model of MI at different time periods (day 3 and 28) was also monitored to investigate if treatment of the animals with PLGA NP $_{CM}$ led to better cardiac function compared to free CM through the reduction of scar area. All 3 groups had an EF of approximately 30% on day 3 post infarction which indicated a successful acute MI[151]. Moreover as demonstrated in **(Figure 5-8B)**, although there was no significant difference in the EF % between the groups on day 3 post infarction, there was a significant improvement on day 28 for the animals treated with hBMSC CM PLGA NPs in comparison to the animals receiving free CM (48.16 ± 5.7% vs 42.33 ± 4.96%). The empty PLGA NPs group did not show any significant improvement in heart functionality. Thus, the echocardiographic EF% values suggest that the delivery of hBMSC CM using PLGA NPs as a sustained release carrier system can be an effective alternative to the administration of free CM for improved heart function after acute myocardial damage.

5.6 Discussion

The possible use of adult stem cell-based therapy for cardiac tissue regeneration post infarct has been demonstrated in numerous studies previously. These studies show that injecting BMSCs into the myocardium led to augmented novascularization, anti- inflammatory and anti-apoptotic effects [53, 54]. However, the mechanisms underpinning the regenerative nature of these stem cells after administration into the myocardium is still unclear with several hypothesis including lineage specific differentiation, transdifferentiation into cardiomyocytes and the secretion of growth factors or paracrine effects [53, 55].

Evidence supporting the hypothesis that paracrine mediators, i.e. growth factors secreted by the implanted stem cells, contribute to myocardial remodelling via stimulation of angiogenesis and suppression of cardiomyocyte apoptosis is on the rise [8, 10-13]. One study showed that the release of cytokines like hVEGF and hbFGF increased collateral perfusion and remodeling rather than due to direct cell injection at the target site [56, 155]. In another study, Gnecchi et al. further demonstrated significant improvement in heart function in less than 72 hrs after intramyocardial injection of Akt-modified mesenchymal stem cells (MSCs) in an ischemic heart rat model [8, 10]. Improved cardiac function although initially to attributed to cell fusion events were so irregular that it has now been credited to indirect paracrine effects. Nguyen et al. also provided further proof to the paracrine hypothesis through an *in vivo* infarcted rat model, when direct administration of the MSC-derived growth factors led to an increased cardiac function and recovery [145]. Furthermore, *in vitro* results by several groups demonstrate that the stem cell generated CM has the ability to promote HUVEC and vascular smooth muscle cell growth and migration.

The potential application of this stem cell generated growth factor cocktail could eliminate the use of stem cells for regenerative therapy and in turn the complications related to their use such as generation of local immune responses, increase in the release of inflammatory mediators [14], tumor formation and growth [15] and drop in cell viability after injection due to the harsh *in vivo* environmental conditions.

The direct administration of several individual or combination of pro-angiogenic growth factors to promote cardiac regeneration has also been explored in-depth by several groups over the years

[156]. Although, the direct administration of growth factors for instance hVEGF has shown initial success, clinical results are limited due certain shortcomings such as the short half life of the administered protein **[157]**. In a study by Formiga et al. the effectiveness of using PLGA microparticles for controlled delivery of the single pro-angiogenic protein hVEGF has been shown **[18]**. Other studies have also demonstrated the advantage of delivering a combination of growth factors such as hVEGF 165, PDGF-BB and hbFGF using polymeric delivery vehicles for potential application in tissue regeneration **[126, 158]**. In the present study, we propose the application PLGA nanoparticles as carriers for the stem cell-derived growth factors so as to allow a more controlled and extended dose of the bioactive factors to the damaged myocardium. Before *in vivo* experiments were performed in the MI rat model, *in vitro* studies were carried out to observe the efficiency of our system. Through *in vitro* release analysis **(Figure 5-2E)** , we were able to demonstrate that our CM loaded PLGA NPs are capable of providing a sustained release over a 21 day period which is similar to other studies **[148]**. We further demonstrated the functional (mitotic and chemotactic) activity of the released growth factor cocktail using HUVEC proliferation, migration and tube formation assays **(Figure 5-3, Figure 5-4, Figure 5-5)**. The use of this cocktail of stem cell-derived growth factors produces an augmented therapeutic effect as compared to the use of individual proteins as demonstrated through the inhibitory studies involving the addition of anti-VEGF antibody to the CM released from the NPs. Our results show that the procedure did not in fact affect the activity of the growth factors loaded within the NPs and the main reason for this could be the co-encapsulation of BSA during the particle formation which has shown to effectively shield the encapsulated biomolecule from the particle surface and in turn avoid its deactivation during particle preparation[93].

During our vivo experiments, treatment of the infarcted heart with PLGA NP $_{CM}$ demonstrated an augmented cardiac function evident through the echocardiographic data, reduced infarct size, a thicker left ventricular wall and increased angiogenesis and arteriogenesis. These results were significantly better than the administration of free CM done in other studies or PLGA NPs $_{Empty}$ which provided evidence of the enhanced therapeutic effectiveness of using our stem cell-generated growth factor loaded PLGA NP system and the possibility of prospective application of this system to achieve functional improvement after MI. The improved heart function in the PLGA NP $_{CM}$ group can be attributed to the decrease in infarct size and increase in viable mass of heart muscles. Moreover, a significant augment in the vascular density in the peri-infarct area for

the PLGA NP $_{CM}$ group was observed. It is hypothesized that it is due to this increased blood vessel density that led to the prevention of loss of cardiomyocytes and suppression of the progression of post-infarction myocardial failure associated with left ventricular remodeling.

As shown in previous studies, the stem cell-generated growth factor cocktail contains a large spectrum of cytokines [56, 155]. However in our study we did not study the role of each cytokine present in the cocktail and emphasized on hVEGF which has been identified as the key therapeutic trophic factor in cardiac regeneration using stem cell therapy[159]. Several studied have observed that a hVEGF concentration of 40-225pg is sufficient to provide significant protection to the cardiac tissue *in vivo*[146]. Nguyen et al. recently highlighted the benefit of using concentrated stem cell generated growth factors as opposed to the use of unconcentrated stem cell-derived factors which requires multiple injections to achieve the desired therapeutic effect. In our study, we injected concentrated stem cell derived growth factors loaded PLGA NPs containing a hVEGF amount of 400 ng. The use of PLGA NPs to deliver the concentrated growth factors not only allows provides the advantages of protein protection preventing denaturation and controlled and extended delivery but also removes the need for multiple injections for myocardial tissue repair. Our study provides an initial step of proposing a strategy of the use of a polymeric system for stem cell generated growth factor cocktail delivery, however extensive future research into identifying the role played by each cytokine in the cocktail on tissue regeneration is still essential. Such studies would further pave the way for the possibility of using genetically modifying the stem cells to alter the secretion of key pro-angiogenic factors needed in augmented amounts for *in vivo* cardiac tissue regeneration. Furthermore, the release kinetics of several other cytokines present in the stem cell-derived growth factor cocktail from the nanoparticles should also be observed to augment the potential of this therapeutic system. An optimal timing of administration of the NPs also needs to be further investigated so as to achieve improved results.

5.7 Conclusion

In conclusion, our stem cell-derived growth factor delivery system could bring together the advantages of using a cocktail of factors with the benefits of a protected, controlled and prolonged release for potential application in myocardial regenerative therapy without the complications of immune rejection and low viability observed with the use of stem cells.

5.8 Acknowledgement

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5.9 List of Figures and Tables

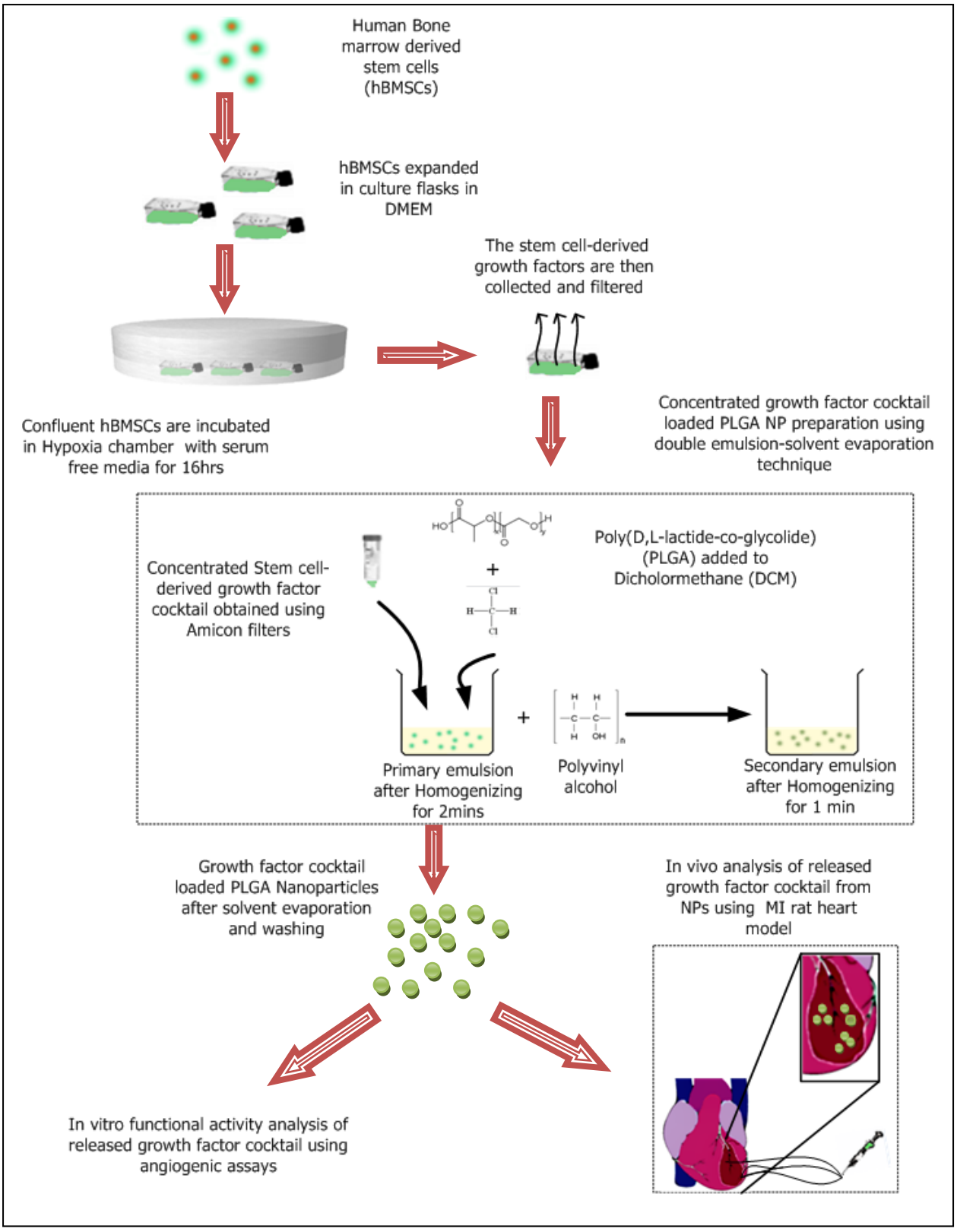

Figure 5-1: Schematic illustrating the entire procedure of stem cell-derived growth factor loaded PLGA NP preparation followed by in vivo studies.

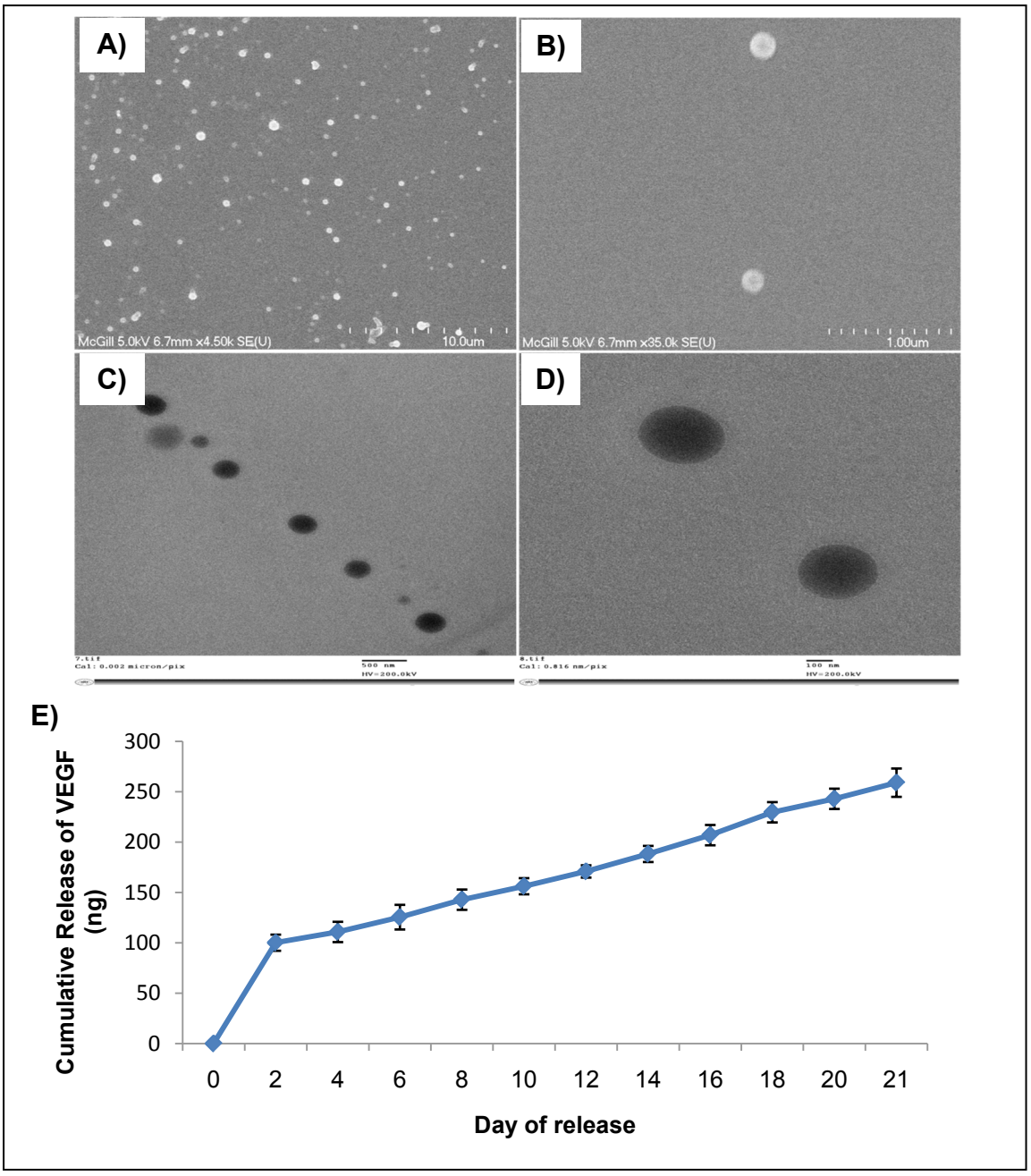

Figure 5-2: Representative images of PLGA nanoparticles prepared using double emulsion solvent evaporation method are shown.

(A, B) SEM and **(C, D)** TEM images **(E)** 21 day *in vitro* release profile of hVEGF present in the hBMSC CM released from the PLGA NPs. Data, which is expressed as cumulative ng of hVEGF released versus time of release, is the mean ± Standard Deviation of three independent experiments determined using ELISA. Scale bars: Bar 10µm **(A)**, 1µm **(B)**, 500nm **(C)**, 100nm **(D)**

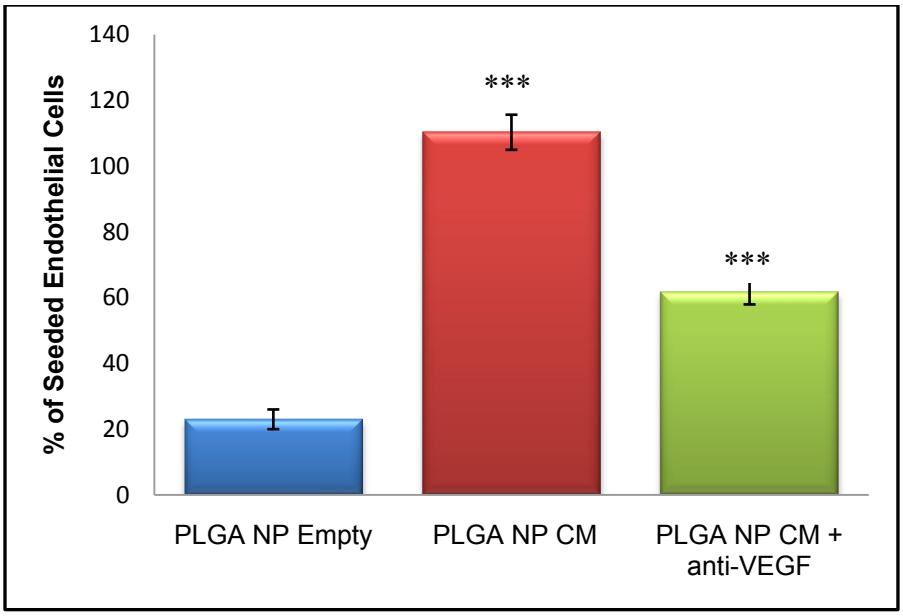

Figure 5-3: Effect of hBMSC CM released on day 4 from the PLGA NPs on HUVEC proliferation.

The cells were incubated with 0.2 ml of the 50% fresh ECM (supplemented with 5% (v/v) FBS without cell growth supplements) and 50% of the supernatant from PLGA NPs loaded with CM (incubated with 10 ml serum free ECM) from day 4, and CM released from the PLGA NPs on day 4 with 10µg/mL anti-hVEGF antibody. Control wells received 0.2 ml of the 50% fresh ECM (supplemented with 5% (v/v) FBS without cell growth supplements) and 50% of supernatant when the empty PLGA NPs were placed in serum free ECM. Briefly, after 96 hours of incubation, data is represented as the mean percentage of endothelial cells on day 4 versus initial cell number (2 x 10^4cells/well) ± Standard deviation of three independent experiments. One way ANOVA analysis: Statistically significant differences between groups compared to the control group are indicated as *** = $p < 0.001$.

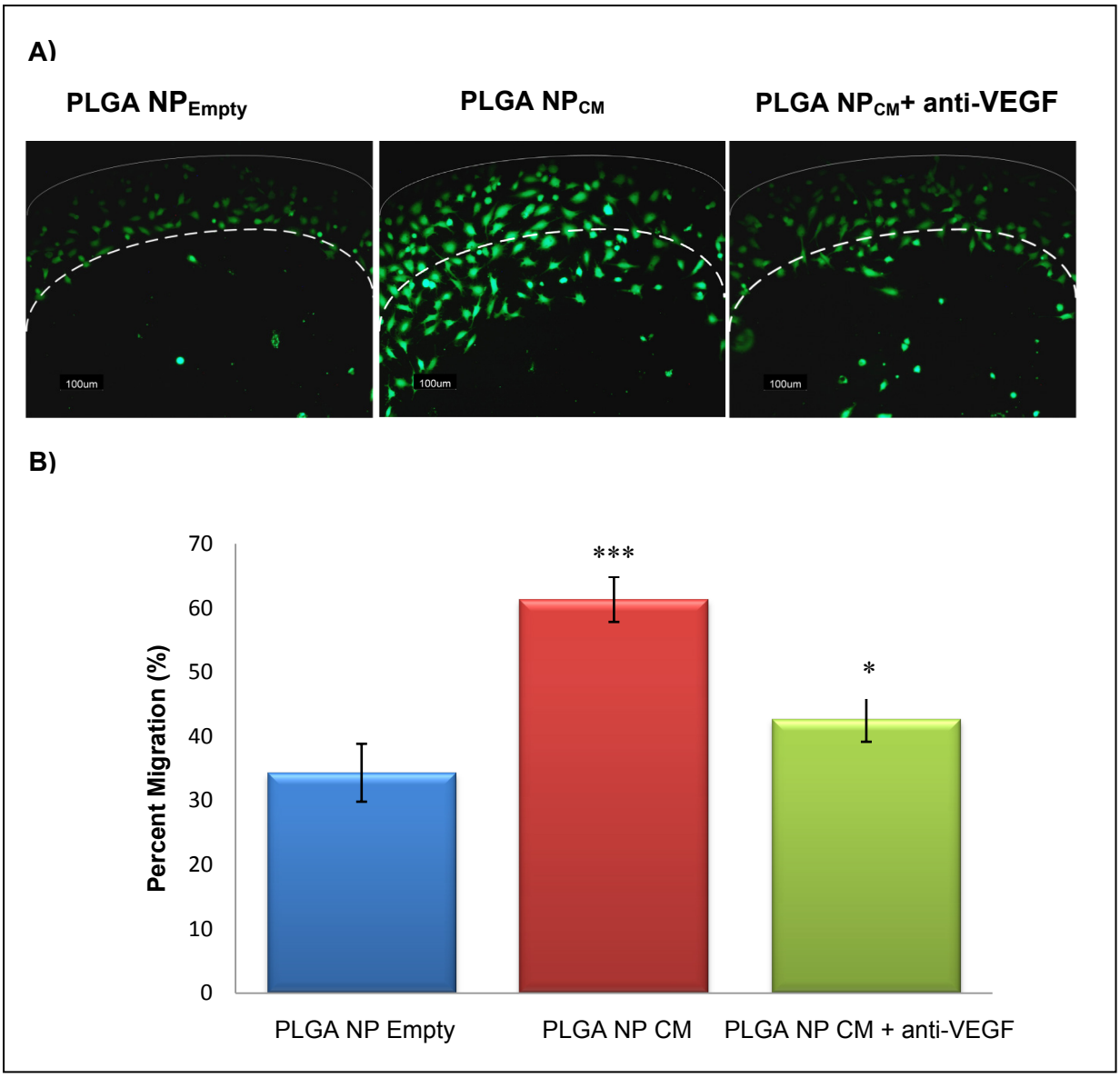

Figure 5-4: Effect of hBMSC CM released on day 4 from the PLGA NPs on HUVEC migration.

The cells were suspended in 50% fresh ECM supplemented with (5% (v/v) FBS without cell growth supplements) and 50% supernatant from PLGA NPs loaded with CM from day 4, and also supernatant with 10µg/mL anti-hVEGF antibody. Control wells received 50% fresh ECM (supplemented with 5% (v/v) FBS without cell growth supplements) and 50% of supernatant when the empty PLGA NPs were placed in 10 ml serum free ECM. **(A)** Cell migration was assessed and percentage of area (initially free of cells marked by the white border line) covered by the migrated cells were analyzed. The dotted line is used to show the increase in HUVEC migration in the other two groups compared the control group with empty PLGA NPs. **(B)** Mean values ± SD are shown. Statistically significant differences between groups compared to the control group are indicated as *** = $p < 0.001$ and * = $p<0.05$. Scale bars: 100 µm.

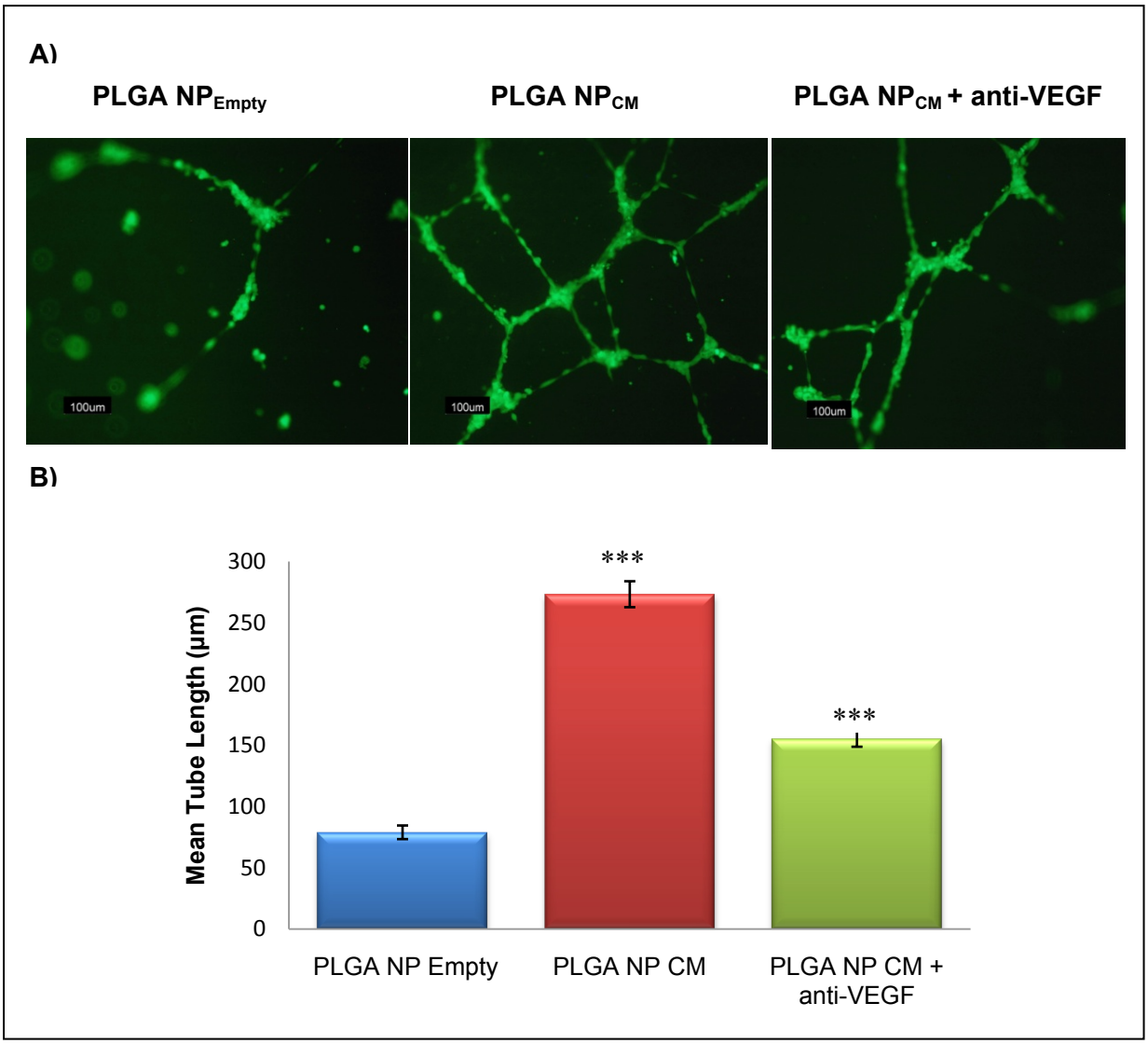

Figure 5-5: **Effect of hBMSC CM released on day 4 from the PLGA NPs on HUVEC tube formation.**

The cells were incubated with 0.2 ml of the 50% fresh ECM (supplemented with 5% (v/v) FBS without cell growth supplements) and 50% of the supernatant from PLGA NPs loaded with CM (incubated with 10 ml serum free ECM) from day 4, and CM released from the PLGA NPs on day 4 with 10µg/mL anti-hVEGF antibody. Control wells received 0.2 ml of the 50% fresh ECM supplemented with 5% (v/v) FBS without cell growth supplements and 50% of supernatant when the empty PLGA NPs were placed in 10 ml serum free ECM. The cells were then incubated with 50µL of 1X Calcein AM for 30 mins at 37^{o}C. **(A)** The endothelial capillary-like tube formation in each well was examined using a fluorescent microscope under 10X magnification. **(B)** The results were quantified as the mean tube length ± Standard Deviation from three independent experiments in µm of the HUVEC-made capillary network. One way ANOVA analysis: Statistically significant differences between groups compared to the control group are indicated as *** = $p < 0.001$. Scale bars: 100µm

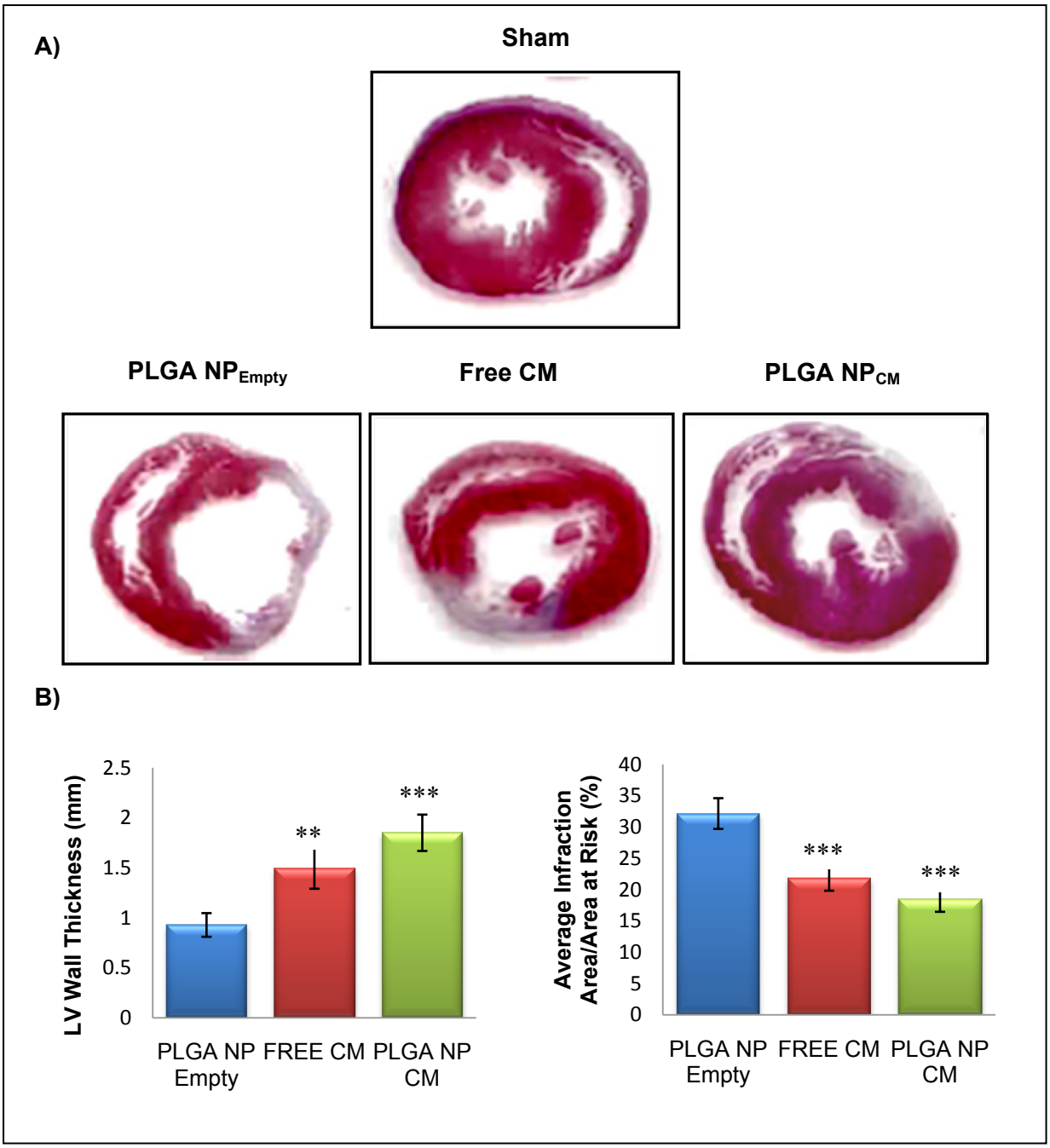

Figure 5-6: Scar area analysis 28 days post infarction.

(A) Representative images of LV myocardial sections stained with Masson's Trichome show the cardiac fibrosis region (in blue). The red area represents the myocardium and the blue area represents extracellular matrix deposition in the scar tissue. The Masson's trichrome stained sections were captured as digital images. The infarct size and wall thickness in the left ventricles at the section of the middle point between ligation and apex were measured. **(B)** Ventricular wall thickness was measured and expressed in mm. Percent infarct size was calculated as infarct area/total LV area x 100 as illustrated in the graph. Statistically significant differences between groups compared to the control group are indicated as *** = $p< 0.001$and ** = $p<0.01$.

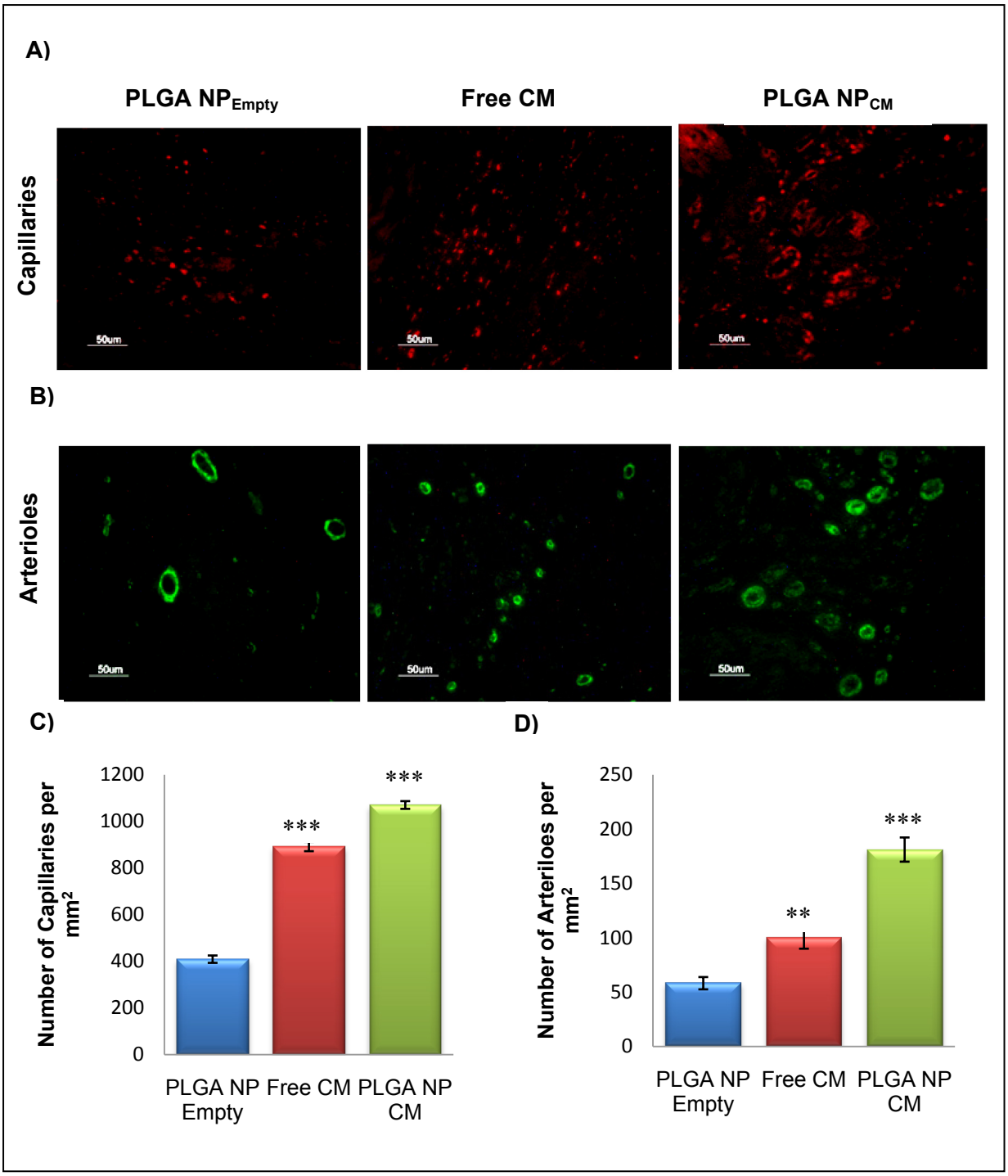

Figure 5-7: Effect of PLGA NPs $_{CM}$ on angiogenesis and arteriogenesis in the peri-infarcted areas.

Immunohistological staining of **(A)** PECAM to detect endothelial cells and **(B)** smooth muscle α-actin to detect smooth muscle cells in all three groups. Quantification of number of vessels/ mm^2. **(C)** Capillary and **(D)** Arteriole density. Data are expressed as mean density ± Standard Deviation. Statistically significant differences between groups compared to the control group are indicated as *** = $p< 0.001$and ** = $p<0.01$. Scale bars: 50 µm

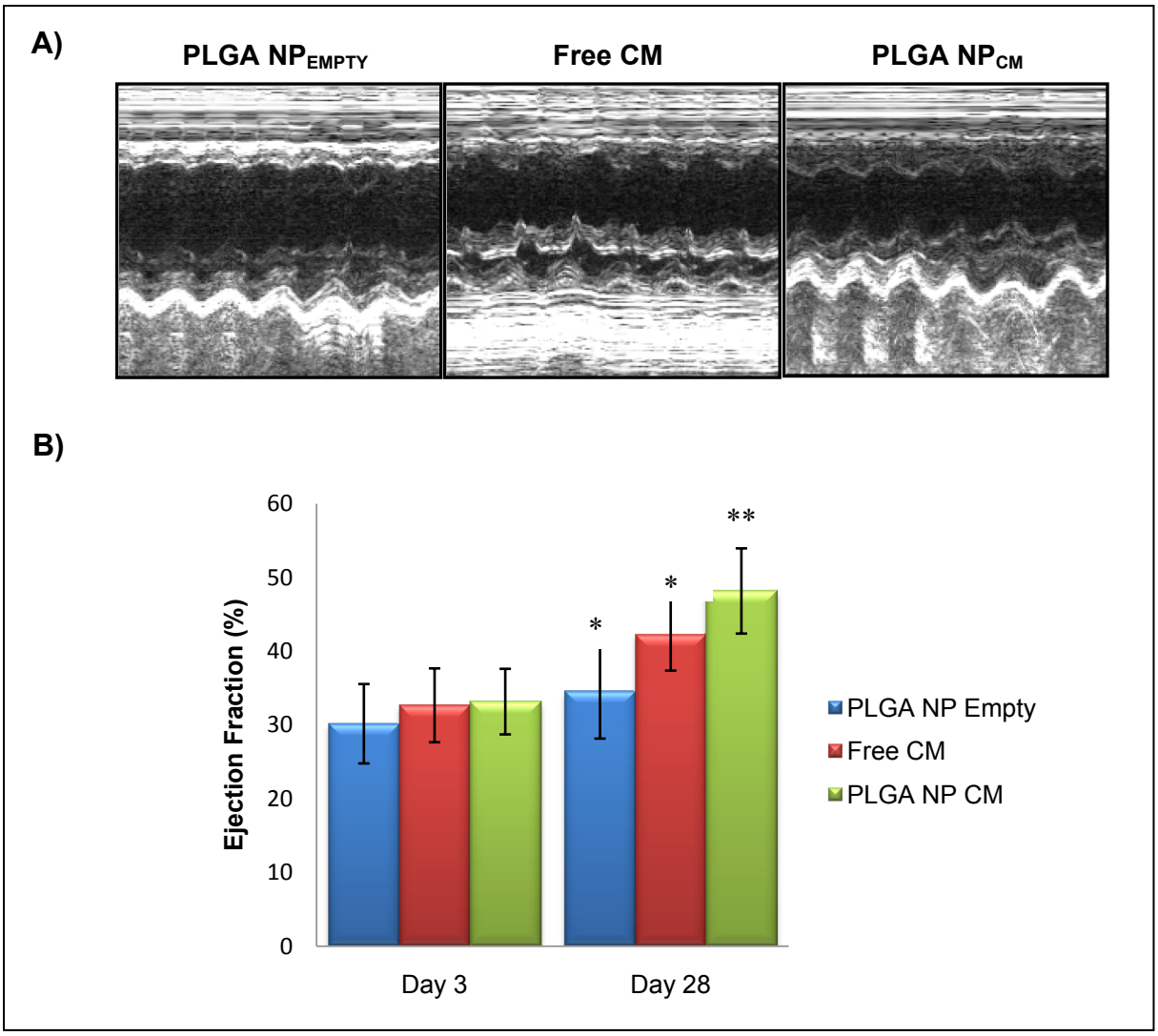

Figure 5-8: Echocardiography data. Effect of treatment with the 3 groups on cardiac function was analyzed over 28 days post infarction.

(A) M-mode echocardiograms of the 3 groups and sham for day 28. **(B)** The myocardial EF% for all three groups was monitored at day 3 and 28 after transplantation as shown in graph. Data is represented as mean EF% values ± Standard Deviation. Statistically significant differences between EF% of a group on day 28 compared to the same group on day 3 are indicated as ** = $p<0.01$ and * = $p<0.05$.

6 Controlled delivery of adipose stem cell derived condition media using polymeric microparticles embedded in hydrogel for effective angiogenic therapy: preparation and *in vitro* analysis

Research article

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6.1 Preface

In chapter 5, the concept of using a continuous delivery particle system encapsulating the stem cell derived growth factor cocktail for a protected and sustained release of the factors was assessed both through *in vitro* and *in vivo* analysis. This chapter demonstrates the possibility and advantage of embedding the loaded controlled delivery polymeric particle systems into a hydrogel matrix. PLGA particles, due to their small size and mobility, do not stay at the delivered site for long and are in fact susceptible to phagocytosis by macrophages. Furthermore, bone marrow derived stem cells are difficult to harvest and grow in large numbers. To answer these issues, PLGA MPs loaded with growth factors harnessed from the easily available adipose derived stem cells were developed and integrated into a collagen matrix to provide a sustained, controlled and extended release of growth factor cocktail for more enhanced tissue regeneration. The *in vitro* release profiles of the two key angiogenic mediators, hVEGF and hbFGF released from the composite collagen matrix embedded with PLGA MPs was studied over a three week period. To determine if the biofunctionality of the encapsulated GFs had been retained through the development of the delivery system, *in vitro* angiogenic assays using endothelial cells were performed.

6.2 Abstract

During the past years, intense research has demonstrated the possibility of using stem cells for several wound healing and tissue regeneration applications. However, recent studies provide evidence that stem cell secreted growth factors (GF) could in fact be the main mediators of regeneration during stem cell therapy. Through the use of GFs or conditioned medium (CM) generated from the stem cells under hypoxic conditions, containing a cocktail of over-expressed GFs such as the key pro-angiogenic growth factors like hVEGF and hbFGF, the current drawbacks faced during the direct application of stem cells such as reduction of cell viability and local immune responses could be overcome. Furthermore, multiple injections of stem cells are required to achieve even modest success. Now, with growing evidence of the potential use of stem cell CM towards tissue regenerative therapy, the need for an efficient delivery system allowing controlled and sustained release of the factors in addition to providing protection from the external *in vivo* environment becomes evident. In our study we propose the development of a three dimensional delivery vehicle consisting of PLGA MPs embedded into a collagen matrix and loaded with human adipose stem cell (hASC) generated GFs for potential -wound healing applications. Release studies illustrate that by day 21 only 52.2 ± 4.8 % of hVEGF and 38 ± 5.1 % of hbFGF present in the hASC-CM were released from the MP/Coll matrix. Functional activity of the released hASC-CM was confirmed using *in vitro* angiogenic assays and an augmented effect was observed in the presence of released hASC-CM on HUVEC proliferation, migration and apoptosis.

Keywords: Stem cells, growth factors, microparticles, collagen hydrogel, tissue engineering, regeneration therapy, angiogenesis, controlled release

6.3 Introduction

The main objective of stem cell therapy is to promote healing of the damaged organ tissue and in turn restore functionality via direct transplantation of adult stem cells at the target site [51]. Numerous studies using stem cells, essentially bone marrow derived stem cells (BMSCs), have repeatedly shown to significantly improve functionality of the injured organ through mechanisms including differentiation, cell fusion and paracrine effects or secretion of cytokines [52]. However, controversy over the mechanisms underlying the therapeutic effects of stem cells has arisen with recent studies indicating that paracrine mediators, i.e. cytokines or growth factors

secreted by the implanted cells, contribute chiefly to tissue regeneration and repair [8, 10-13]. Cell fusion events associated with improved organ function were observed but these cellular fusion and diffusion events were so irregular that the improved heart function has been credited to indirect paracrine effects rather than cardiac tissue regeneration. Moreover, it has been demonstrated that under hypoxic and serum starvation culture conditions, such as the microenvironment found in ischemic heart tissue, a wide variety of biologically active factors known to induce cardiovascular signaling are secreted by Mesenchymal stem cells (MSCs) and found in the MSC-conditioned culture medium (MSC-CM) [56]. Several studies have shown that this MSC-CM promotes the *in vitro* proliferation and migration of endothelial cells and vascular smooth muscle cells. The use of this stem cell generated CM could evade the concept of stem cell administration for tissue regenerative purposes and subsequently the related drawbacks to their use: It has been shown that the transplantation of MSCs cause local immune responses and increase the release of inflammatory mediators in turn disrupting homeostasis within tissue [14]. In addition, the ability of these stem cells to suppress immune responses could promote tumor formation and growth [15]. It has also been shown that cell viability reduces after injection due to the harsh *in vivo* environmental conditions.

Although BMSCs have widely been applied in clinical trials, their availability is limited and they are difficult to harvest and expand *in vitro*. Human adipose derived stem cells are multipotent cells that can easily be harvested from a discarded product (lipoaspirate) obtained during liposuction and contrary to BMSCs, are present in large numbers [160]. Now recent studies have provided evidence that the secretion of growth factors or cytokines by hASCs can produce therapeutic effects such as increased angiogenesis and reduction of apoptosis leading to accelerated tissue regeneration and healing [161]. In fact, several studies have showed that under hypoxic conditions, the paracrine effects of hASCs are augmented and an enhanced secretion of multiple proangiogenic and anti-apoptotic growth factors such hVEGF and hbFGF was observed [155, 161].

Although several studies demonstrate the potential of using stem cell derived CM for wound healing and regenerative therapy, successful tissue regeneration is dependent on the controlled and continuous delivery of growth factors to the tissue cells producing a growing awareness for the requirement of sophisticated drug delivery devices mimicking the endogenous production

profiles of growth factors during native tissue regeneration. Furthermore, there have been several studies where single or dual pro-angiogenic proteins have been directly injected as a bolus, and although initial results have been promising, the effect is short lived due to protein instability or denaturation after injection [16, 17]. The application of composite matrices comprising of a collagen matrix incorporating PLGA MPs loaded with growth factors has shown tremendous potential in regenerative therapy [66, 93, 162]. Numerous studies also show that PLGA MPs not only protect the growth factors from the harsh *in vivo* environment and prevent protein deactivation but also allow controlled release of the growth factors to the site without rapid diffusion [66, 126]. However, these PLGA MPs, due to their small size and mobility, do not stay at the delivered site for long and are in fact susceptible to phagocytosis by macrophages [163]. To overcome this problem these PLGA MPs have been incorporated into matrices to enable a more controlled, continuous and extended release of growth factors at the target wounded site. Collagen forms the main component of the native extracellular matrix in all connective tissues and because of its properties of good biocompatibility and low antigenicity has been used widely in matrix development for GF delivery in wound healing and tissue regenerative therapy [164].

In this study, as illustrated in **(Figure 6-1)** we prepared a delivery system consisting of PLGA MPs loaded with hASC-CM integrated into a collagen matrix to provide a sustained, continuous and extended release of growth factor cocktail for more enhanced tissue regeneration. The *in vitro* release profiles of the two key angiogenic mediators, hVEGF and hbFGF released from the composite collagen matrix embedded with PLGA MPs was studied over a three week period. The functional activity of the released CM was assessed through *in vitro* angiogenic assays using HUVECs.

6.4 Materials and Methods

6.4.1 Materials

Poly(D,L-lactide-co-glycolide) (PLGA, RESOMER RG 504H, Sigma-Aldrich, USA), dicloromethane (DCM, ACP Chemical Inc., Montreal, Quebec), Poly(vinyl alcohol) (PVA, Sigma-Aldrich, USA), Phosphate Buffered Saline (PBS, Invitrogen, GIBCO, NY, USA), Nile red (Fluka Analytical, UK), Human vascular endothelial growth factor (hVEGF) ELISA assay (R & D systems, Minneapolis, Minnesota, USA), Human basic fibroblast growth factor (hbFGF) ELISA assay kit (RayBiotech Inc, USA), CellTiter 96® AQ_{ueous} Non-Radioactive Cell

Proliferation MTS Assay (Promega, Madison, Wisconsin, USA). hVEGF and hbFGF Antibodies (Santa Cruz Biotechnology, California, USA), Albumin from bovine serum (BSA, Sigma-Aldrich, USA).

6.4.2 Cell culture

Human adipose derived stem cells (hASC, Invitrogen, USA) and Human umbilical vein Endothelial cells (HUVEC, Sciencell, Carlsbad, California, USA) were cultured and expanded in 75cm^2 cell culture flasks (Corning, NY, USA) at 37^{o}C and under 5% CO_2 atmosphere. hASCs were grown in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, GIBCO, NY, USA) supplemented with 10% (v/v) Fetal bovine albumin (FBS) and HUVECs were grown in Endothelial cell medium (ECM) (Sciencell, Carlsbad, California, USA) supplemented with 5% (v/v) FBS and cell growth supplements as per supplier's instructions. The cells in passages 4 and 5 were used in the experiments.

6.4.3 Generation of CM from human adipose derived stem cells

After the hASCs had reached a confluency of approximately 90-95%, the DMEM with 10 % FBS was replaced with serum free DMEM and the cells were then cultured under hypoxic conditions (5% CO_2 and balanced N_2) in a Billups-Rotenberg modular incubator chamber. The incubator is flushed for 5-10 minutes at 10-15 l/min and placed in the 37^{o}C incubator for 72hrs. At the end of the incubation period, the conditioned medium from the hASCs cultured under hypoxic conditions was collected and filtered through a 0.22 μm syringe filter. The proteins were then concentrated using Millipore Amicon centrifuge filters (Millipore, Bedford, USA).

6.4.4 Preparation of hASC-CM loaded MPs

PLGA MPs loaded with hASC-CM were prepared by double emulsion-solvent evaporation technique as previously described [93]. Briefly, the generated concentrated hASC-CM was added to 50mg PLGA dissolved in 1 ml DCM. BSA was also added to the solution to maintain stability of the growth factors during encapsulation procedure as done before. The primary emulsion was generated by a high speed homogenizer (PowerGen 125, fisher Scientific, USA) operating for 2mins. The double emulsion was obtained by adding the emulsion to 10ml of 0.5% (w/v) PVA and homogenizing again for 1 min. To remove the organic solvent, the resultant W/O/W emulsion was then placed under magnetic stirring for 3 hrs at room temperature. After 3hrs, the hardened PLGA MPs were then washed three times with distilled water by

centrifugation. The MPs were prepared in three different batches and frozen at -20^{o}C for further use. Empty PLGA MPs were prepared as negative control and fluorescent PLGA MPs loaded with 10mg of Nile red (Sigma-Aldrich, UK) were also prepared for characterization studies.

6.4.5 Loading efficiency of hVEGF and hbFGF present in hASC-CM within MPs

Encapsulation efficiency of hVEGF and hbFGF present in hASC-CM loaded within the MPs was determined as previously performed [93]. The hASC-CM MPs were completely degraded by adding the particles to 1ml of 0.5N NaOH under stirring. After 24, the solution was centrifuged and the amount of hVEGF and hbFGF was analyzed using hVEGF and hbFGF ELISA assay kits according to manufacturer instructions. Results were expressed as loading efficiency (ratio of actual to theoretical loading x 100) ± standard deviation of values collected from three different batches.

6.4.6 Preparation of MP/Coll matrix

The MP/Coll matrix was prepared by a previously described method [66]. Briefly, purified collagen (6.4 mg/ml, Nutragen, Inamed Biomaterials) was diluted to 4.8mg/ml and the PLGA MPs were added to the pre-gelled collagen to get a final concentration of 150 ± 4.7 ng hVEGF. In order to obtain a firm gel, the MP/Coll solution was then added to a 6 well culture plates and incubated at 37^{o}C for approximately 45min. Fluorescent MP/Coll matrices were prepared using the same protocol by adding nile red loaded MPs to the pre-gelled collagen solution.

6.4.7 Characterization of MPs and MP/Coll matrix

Scanning Electron Microscopy (SEM) was used to observe the external shape and morphological characteristics of the prepared PLGA MPs. The MPs were first dispersed in ddH_2O and then dried with natural convection under room temperature. The MP/Coll matrix was examined by adding a drop of the solution on to a cover slip and allowed to dry. The samples were then examined under S4700 FEG-SEM (Hitachi, Oakville, Ontario, Canada).

Transmission Electron Microscopy (TEM) was used to obtain the size characterization by suspending the PLGA MPs in 1X PBS and observed on CM200 FEG-TEM (Philips, Markham, Ontario, Canada).

The Atomic Force Microscopy (AFM) images were produced with Nanoscope III AFM (Digital Instruments, USA) using silicon cantilever in tapping mode and analyzed using Nanoscope

software version 5.12r5. The PLGA MPs were vortexed and 1µl of the sample was dropped on to the surface of the discs and dried using conventional method.

Images of the collagen matrix embedded with nile red loaded PLGA microspheres were taken using a fluorescent microscope in both bright field and fluorescent field under 30X magnification.

6.4.8 In vitro release of hVEGF and hbFGF present hASC-CM from PLGA MPs and PLGA MP/Coll matrices

The *in vitro* release profiles of two main angiogenic proteins present in hASC-CM, hVEGF and hbFGF, from only PLGA MPs and from the MP/Coll composite was investigated for 21 days. The hASC-CM loaded MPs and the MP/Coll composite matrices were prepared according to the protocol described earlier and incubated with 2ml serum free ECM with 1% penicillin/streptomycin at 37^{o}C under constant shaking. At predetermined time intervals, 0.2ml of the released medium was removed and equal amount of fresh ECM was added back to maintain constant volume. The concentration of hVEGF and hbFGF present in the hASC-CM released into the medium was determined using hVEGF and hbFGF ELISA assay kits. Data represents cumulative ng of either hVEGF or hbFGF released (mean ± Standard Deviation of three independent experiments) versus time of release.

6.4.9 HUVEC proliferation assay

The bioactivity of hVEGF and hbFGF present in the released hASC-CM from the MP/Coll composite system was assessed *in vitro* by determining the proliferative capacity of HUVECs. CellTiter 96® AQueous Non-Radioactive Cell Proliferation MTS Assay kit was used to evaluate the proliferation of the HUVECs in response to the added supernatant containing hASC-CM released from the matrix and negative control. The HUVECs were seeded at a density of 2x10^{4} cells/well in a 96 well plate in ECM supplemented with 5% (v/v) FBS and cell growth supplements. After 24 hours, the ECM was removed and the cells were then incubated for 96 hours with 0.2 ml of 50% fresh ECM supplemented with 5% (v/v) FBS without cell growth supplements and 50% of the supernatant from each of the following 5 groups: Group1 containing hASC-CM released from the MP/Coll matrix (incubated with 2ml serum free ECM) on day 4, group 2 containing hASC-CM with 10µg/mL anti-hVEGF antibody, group3 containing hASC-CM with 5µg/mL anti-FGF antibody, group 4 containing hASC-CM with both anti-hVEGF and

anti-FGF antibodies. Control wells received 0.2 ml of the 50% fresh ECM supplemented with 5% (v/v) FBS without cell growth supplements and 50% of supernatant when the empty PLGA MP/Coll composites were placed in 2 ml serum free ECM. Results illustrated as the mean percentage of endothelial cells on day 4 versus initial cell number (2 x 10^4cells/well) (± Standard deviation) were determined by MTS assay which was performed as per the manufacturer's protocol. Briefly, after 96 hours of incubation, the HUVECs were washed with PBS and fresh ECM was added to the wells. This was followed by the addition of 20µl of MTS solution to each well and the cells were incubated in 37^{o}C for 4hrs under 5% CO_2 atmosphere. The absorbance was measured at 490 nm using the 1420-040 Victor3^{TM} Multilabel Counter (Perkin Elmer, Woodbridge, Ontario, Canada). Results from the ELISA were used to determine the concentration of hVEGF and hbFGF present in hASC-CM released into the supernatant.

6.4.10 Wound Healing Assay

The potential of hASC-CM released from the MP/Coll complex on day 4 in assisting HUVECs towards wound healing was assessed using the classic wound healing assay protocol as described previously [165]. HUVECs were first seeded in a 24 well plate and grown to reach a confluency of 90-95% and were then placed under serum starvation conditions (1% FBS) for 24 hrs. The cell monolayer was then scraped using a p200 pipet tip to create an even scratch through the seeded cells. The cells were washed once with PBS to remove the debris and smooth the scratch edges and then incubated for 12 hrs with 0.2 ml of 50% fresh ECM supplemented with 5% (v/v) FBS without cell growth supplements and 50% of the supernatant from each of the following 5 groups: Group1 containing hASC-CM released from the MP/Coll matrix (incubated with 2 ml serum free ECM) on day 4, group 2 containing hASC-CM with 10µg/mL anti-hVEGF antibody, group3 containing hASC-CM with 5µg/mL anti-FGF antibody, group 4 containing hASC-CM with both anti-hVEGF and anti-FGF antibodies. Control wells received 0.2 ml of the 50% fresh ECM supplemented with 5% (v/v) FBS without cell growth supplements and 50% of supernatant when the empty PLGA MP/Coll composites were placed in 2 ml serum free ECM. To determine the number of cells (all groups) that had migrated across the starting scratch point after 12 hrs, the cells were washed with PBS and fixed with 4% paraformaldehyde (Sigma). Crystal Violet was then added to each well to stain the cells. The wells were observed under 20X magnification and Image J software was used and the number of cells which had moved across the starting scratched lines were measured for all groups.

6.4.11 Anti-apoptosis effect of hASC-CM on HUVECs

The ability of hASC-CM released from the microsphere/collagen matrix to prevent HUVEC apoptosis was studied next. The MP/Coll matrices were placed into a 6 well plate and incubated at 37^{o}C under constant shaking with 2 ml serum free ECM added to each well. At predetermined time points, 0.4ml of supernatant was removed and frozen at -80^{o}C and the same amount of medium was added back to keep constant volume in the wells. HUVECs (seeding density 2 X 10^4 cells/well) were placed into a 96 well plate in ECM (supplemented with 5 % FBS and cell growth factors) and incubated for 24hrs at 37^{o}C. After 24hrs, the ECM was removed and the HUVECs were incubated for 96hrs with 0.4 ml of supernatant from each of the following 5 groups: Group1 containing hASC-CM released from the MP/Coll matrix (incubated with 2 ml serum free ECM) on day 4, group 2 containing hASC-CM with 10µg/mL anti-hVEGF antibody, group3 containing hASC-CM with 5µg/mL anti-FGF antibody, group 4 containing hASC-CM with both anti-hVEGF and anti-FGF antibodies. Control wells received 0.4 ml of supernatant when the empty PLGA MP/Coll composites were placed in 2 ml serum free ECM. After 96 hrs, the cells were washed with PBS and the number of floating cells was counted. To determine the number of apoptotic cells, MitoTracker® Red CMXRos Kit and DAPI nucleic acid staining (Invitrogen, Burlington, Canada) were used according to manufacturer's instructions. The cells were counted using fluorescence microscopy and the results were illustrated as the percentage of apoptotic adherent cells and floating cells in each well. Three independent experiments were performed.

6.4.12 Statistical Analysis

Data are presented as mean ± Standard Deviation from independent experiments. Each experiment was tested in triplicate. The bioactivity of the released condition media was analyzed using Student's t-test for statistical comparisons between pairs of samples, where "*" indicates the statistical difference between the group with released CM and the other groups ($*p < 0.05$).

6.5 Results

6.5.1 Regulation of hVEGF and hbFGF secretion under Hypoxic conditions

hASCs were incubated for 72 hrs with DMEM with 5% FBS and under normoxic conditions secreted significant amounts of hVEGF and hbFGF as demonstrated by their respective ELISA assay kits but there was a prominent increase in the amount of hVEGF and hbFGF generated in

the hypoxic CM. ELISA results demonstrate that there was almost a 4-fold up-regulation in hVEGF secretion under hypoxic conditions (3.9 ± 0.6ng/10^6cells) in contrast to that under normoxic conditions (1.2 ± 0.3ng/10^6cells). hbFGF secretion was up-regulated by approximately 2-folds under hypoxic conditions (0.29 ± 0.03ng/10^6 cells) compared to that under normoxic conditions (0.13 ± 0.02ng/10^6cells).

6.5.2 Characterization of MPs and MP/Coll matrix

The PLGA MPs prepared using double emulsion-solvent evaporation technique had an average diameter ranging between 1 to 35µm. BSA was co-encapsulated with the hASC-CM as it has shown to prevent protein deactivation during the particle formulation procedure [93]. To determine the amount of hVEGF and hbFGF present in hASC-CM loaded within the PLGA MPs, the particles were dissolved in NaOH. The encapsulation efficiency was found to be 54.5 ± 9.3% and 88.7 ± 7.5% respectively, as determined by the respective ELISA assay kits. The estimated dose of hVEGF in the hASC-CM encapsulated in the PLGA MPs was 3.02 ng per mg of polymer. SEM, TEM and AFM images **(Figure 6-2A,B,C,D)** showed that the hASC-CM loaded PLGA MPs were perfectly spherical in shape with an intact surface devoid of wrinkles or cracks.

In fact, it was verified through SEM images as shown in **(Figure 6-2F)** that the PLGA microspheres formed an integral part of the collagen matrix with no significant effect on microsphere morphology after incorporation. The incorporated PLGA microspheres were observed to be uniformly distributed throughout the collagen matrix. **(Figure 6-2E)** illustrates the surface morphology of the collagen matrix without PLGA microspheres. To demonstrate how the PLGA polymeric structure shields the loaded growth factors from degradation due to harsh external conditions and how the encapsulated agent is uniformly distributed within the polymeric structure, nile red loaded PLGA microspheres were formed. **(Figure 6-2G,H)** illustrates images of the collagen matrix embedded with nile red loaded PLGA microspheres taken using a fluorescent microscope under bright field and fluorescent field.

6.5.3 In vitro release of hVEGF and hbFGF present in hASC-CM from PLGA MPs and MPs/collagen matrix:

The *in vitro* release kinetics profile of two main angiogenic proteins present in hASC-CM, hVEGF and hbFGF, from only PLGA MPs and from the MP/Coll composite was investigated

for 21 days as illustrated in **(Figure 6-3)**. For both delivery systems, the protein release curve was characterized by two phases: an initial burst phase followed by a slower constant release over time. However, it was observed that there was a significant reduction in the burst release of the proteins from the MP/Coll composite in comparison to the release from the MPs not incorporated into collagen matrix with the burst release of hVEGF from the MP/Coll composite being 23.68 ± 4.2 ng vs 45.78 ± 3.15 ng from PLGA MPs and hbFGF from the MP/Coll matrix being 2.08 ± 0.45 ng vs 3.98 ± 0.41 ng from only PLGA MPs. **(Figure 6-3A)** also illustrates that by day 21 of the release study, 75.2 ± 9.2 % of encapsulated hVEGF had been released from only PLGA MPs into the external medium. In contrast, the release from the MP/Coll matrix was observed to be slower and more controlled and by day 21 only 52 ± 4.8 % of hVEGF had been released demonstrating a potentially extended release profile. As seen in **(Figure 6-3B)**, by day 21, 38 ± 5.1 % hbFGF had been released from MPs/collagen composite which was much lower compared to the 53 ± 3.8 % released by only PLGA MPs not embedded in the collagen matrix.

6.5.4 Effect of hASC-CM on HUVEC proliferation

In our study, we measured the effect of supernatant containing hASC-CM released from the MP/Coll matrix on the survival and growth of human endothelial cells, which is considered crucial to the successful progression of angiogenesis [154]. It is known that endothelial cells, typically inactive in existing vessels, proliferate on stimulation by angiogenic factors. CellTiter 96® AQueous Non-Radioactive Cell Proliferation MTS Assay kit was used to access the *in vitro* bioactivity of encapsulated hASC-CM released over time from the MP/Coll matrix. Results illustrated **(Figure 6-4)** as the mean percentage of endothelial cells on day 4 versus initial cell number (2 x 10^4cells/well) (± Standard deviation) were determined by MTS assay, where proliferation with supernatant from CM loaded MP/Coll matrix was most significant compared to the other groups (* $p < 0.05$). It was observed that the HUVECs incubated with 50% ECM (5 % FBS without cell growth supplements) and 50% supernatant from MP/coll matrix (containing hVEGF amount 2.6 ± 0.5 ng) significantly enhanced the growth of HUVECs compared to wells receiving supernatant from empty MPs/collagen matrix without CM (94.5 ± 6.5 % vs 25.4 ± 2.5 % of seeded HUVEC cells). The supernatant from empty composites did not significantly affect HUVEC proliferation indicating that it had no toxic effect on the cells. In order to demonstrate the individual effect of the angiogenic proteins, hVEGF and hbFGF on HUVEC proliferation, antibodies against the respective proteins were added. A significant drop in HUVEC cell growth

was observed in the presence of anti-hVEGF (47.5 ± 4.3 %). In contrast, the addition of anti-hbFGF did not cause a considerable difference to HUVEC proliferation (81.3 ± 6.2 %) as compared to the absence of hVEGF. To show the combined effect of these two key pro-angiogenic proteins on cell growth, anti-hVEGF and anti-hbFGF antibodies were added and a major drop in HUVEC proliferation (32.4 ± 3.5 %) was observed proving the impact of these growth factors in therapeutic angiogenesis. These inhibitory studies further demonstrated that despite the presence of blocking antibodies against hVEGF and hbFGF, which are key angiogenic proteins, there still remained a considerable mitogenic effect on the HUVEC number over the control demonstrating the influence of the numerous growth factors present in the stem cell generated cytokine cocktail.

6.5.5 Effect of hASC-CM on HUVEC wound healing

We used the wound healing scratch assay to access the ability of the supernatant containing hASC-CM released from the MP/coll matrix to assist in wound healing after trauma has been caused to the cells. Wound closure is an essential characteristic of HUVECs in the presence of angiogenic growth factors and we aim to augment this phenomenon through the continuous availability of a cocktail of pro-angiogenic factors at the desired site. Confluent HUVECs were serum starved and then scraped with a pipette tip to create a wound. As illustrated **(Figure 6-5)**, a greater wound healing effect was observed when HUVECs were incubated with 50% ECM (5% FBS without cell growth supplements) and 50% supernatant with hASC-CM released from the MP/Coll matrix (hVEGF amount 2.6 ± 0.5 ng) as compared to the cells receiving unconditioned supernatant from empty MP/Coll matrix (85.3 ± 3.9 % vs 15.2 ± 2.3). Wound closure was drastically inhibited in the presence of antibodies (Anti-hVEGF antibody: 47.5 ± 5.2 % and Anti-hbFGF antibody: 73.4 ± 3.2 %) when compared to addition of supernatant without antibodies. These results highlight the strong chemotactic ability of hVEGF and hbFGF to stimulate HUVEC movement during wound healing. Data is representative mean ± Standard Deviation of 3 independent experiments. Our data thus clearly demonstrates that, hASC-CM released from the MP/Coll matrix has the potential to promote HUVEC wound healing with the number of cells that migrated in the presence of released hASC-CM being significantly higher than the other groups (* $p < 0.05$).

6.5.6 Anti-Apoptotic effect of hASC-CM on HUVECs

In order to demonstrate the anti-apoptotic effect of hASC-CM released from the MP/Coll composite, serum deprivation method was used. It is known that under conditions of serum deprivation, the seeded HUVECs tend to undergo rapid apoptosis and get detached from the culture plates. Although several studies have demonstrated the anti-apoptotic effect of various doses of individual proteins on HUVECs, the use of a cocktail of growth factors in our study clearly shows an improved effect in comparison to the delivery of individual proteins. The apoptotic cells in the adherent cells was determined using MitoTracker® Red CMXRos kit and DAPI nucleic acid staining and counted using fluorescence microscope as shown in **(Figure 6-6)**. **(Figure 6-6F)** demonstrates that apoptosis in the presence of supernatant containing hASC-CM significantly reduced to 26.3 ± 3.4 % while the control wells with supernatant from the empty MP/Coll matrix showed an apoptosis of 95.6 ± 3.1 %. The individual anti-apoptotic effect of hVEGF and hbFGF on HUVECs was observed by the addition of neutralizing antibodies to the hASC-CM containing supernatant where the apoptosis increased to 46.41 ± 3.5 % in the presence of anti-hbFGF antibody and further increased to 62.3 ± 4.1% in the presence of anti-hVEGF neutralizing antibody.

As seen in **(Figure 6-6B)**, the anti-apoptotic effect of hASC-CM on the seeded HUVECs in comparison to the control wells **(Figure 6-6A)**, containing supernatant from empty composites is clearly visible. A distinct change in the morphology of the cells undergoing apoptosis (indicated by yellow arrows) was observed in comparison to live cells (indicated by white arrows). The number of apoptotic cells is significantly reduced when the HUVECs are incubated with supernatant with CM. The anti-apoptotic effect of the hASC-CM released into the supernatant on the seeded HUVECs is demonstrated which illustrates the significantly lower apoptosis of HUVECs in comparison to the other groups (* $p < 0.05$). The individual effect of hVEGF and hbFGF in the CM is also illustrated in **(Figure 6-6C, D)** through the addition of neutralizing antibodies. **(Figure 6-6E)** shows that in the presence of antibodies against both hVEGF and hbFGF added to the hASC-CM containing supernatant, the percent apoptosis is still lower than the control demonstrating the anti-apoptotic effect of the other cytokines present in the CM. Three independent experiments were performed and the mean± Standard Deviation are represented in the graphs.

6.6 Discussion

In recent years, several groups have demonstrated the therapeutic potential of using stem cell therapy towards wound healing and tissue regeneration. Studies with bone marrow stem cells in particular have shown promising results due to existing experience with their use [166]. However, mechanisms underlying these effects produced by the administered stem cells are still controversial. For example, BMSCs, known to secrete a wide range of cytokines and growth factors, have been used for cardiac regenerative studies through intramycordial injection into the ischemic heart with significant improvement of the myocardium function as early as 3days after BMSC injection. It is hypothesized that such an early recovery cannot be explained explicitly by *de novo* myogenesis and that the injected stem cells achieve cardioprotection by indirect effects, through paracrine mediators or released growth factors [8, 9]. The study by Kinnard et al. showed that marrow stromal cells enhance collateral perfusion and remodeling, largely mediated through the release of several arteriogenic cytokines like hVEGF and hbFGF rather than direct cell administration at the target site [56, 155]. Nguyen et al. also validated the paracrine hypothesis *in vivo* by directly injecting the MSC-CM into the infarcted heart and showed an augmented myocardial repair and function [145].

Although several studies involving BMSC-CM have emerged, the potential use of paracrine factors secreted by adipose derived stem cells is slowly gaining recognition due to their easy accessibility and simple minimally invasive procedure for harvest from the site [161]. The study also showed that the adipose stem cell conditioned medium generated, significantly increased proliferation and induced an anti-apoptotic effect on endothelial cells which in turn provides evidence of the potential of using hASC-CM as a therapeutic option to promote angiogenesis and enhance wound healing and tissue regeneration.

It is evident through previous work that the stem cell derived growth factor cocktail contains a broad spectrum of factors. In our study, CM was generated from hASCs under hypoxic conditions and the amount of hVEGF and hbFGF present in the CM was identified using ELISA assay kits. We did not examine the role played by each cytokine present in the cocktail, but with numerous upcoming studies proving the key pro-angiogenic and anti-apoptotic potential of these proteins towards improved tissue regeneration and wound healing [167] coupled with significant increase in the production of these proteins during hypoxic conditions in the tissue after stem cell

implantation, emphasis on these two proteins in the current study becomes obvious [9, 161]. With increasing evidence supporting the paracrine hypothesis, the need for an efficient delivery vehicle for these paracrine factors becomes apparent, which forms the basis of this study. The potential of using CM generated from hASCs integrated with tissue engineering principles to develop a suitable continuous delivery system allowing the localized, sustained and extended release of factors for wound healing and tissue regeneration was studied. The development of a system to deliver the stem cell-derived growth factors would help avoid the drawbacks related to the direct use of stem cells such as immune response and decreased cell viability. Moreover, although numerous groups have shown initial success to bolus administration of individual growth factors, this effect is temporary and has highlighted the need for an effective delivery system which protects the injected protein as well as provides a sustained and controlled release at the site [18]. Through our mechanism i.e. encapsulating the stem cell growth factors inside PLGA MPs embedded in a collagen matrix, an augmented regenerative effect was observed mimicking the paracrine effect created by stem cells. Borselli et al. recently showed that incorporation of PLGA microspheres into a collagen matrix enabled preservation of the biological factor loaded within the microspheres as well as spatial and temporal tuning of the protein release pattern [93]. The hASC-CM loaded PLGA MPs developed in our study were incorporated onto a collagen matrix during fabrication and it was observed as seen in **(Figure 6-2)** that uniformly distributed PLGA microspheres formed an integral part of the collagen matrix without deformations in the MP structure. Research has shown that the double emulsion/solvent evaporation technique for particle preparation could lead to protein denaturing [130]. In order to prevent this, BSA was added during MP formulation so as to shield the loaded protein cocktail from the macropore surface and in turn avoid deactivation. Bioactivity of the encapsulated growth factors was retained through particle processing and release which was apparent through the augmented HUVEC proliferation, migration and survival in response to the released hASC-CM.

It was observed as demonstrated in **(Figure 6-3)** that the MP/Coll composite, after the initial burst, extended the *in vitro* release behavior of both the proteins over the three week study period in a controlled and sustained manner. Now, as evident from our release kinetics graphs illustrated in **(Figure 6-3)**, the MP/Coll composite demonstrated a slower and more extended release of

hASC-CM into the supernatant in comparison to the GFs released from MPs not incorporated into a collagen matrix.

The ultimate aim of tissue engineering and regenerative medicine is to provide a stable and continuous supply of bioactive growth factors to the target site promoting healthy tissue healing and repair. In this study, the angiogenic proteins, present in the CM, are known to have a very short half-life and rapidly degrade in vivo [67, 168]. Thus, to evaluate the angiogenic potential of the hASC-CM released from the MP/Coll composite, we used in vitro angiogenic assays: endothelial cell proliferation assay (proliferation), scratch wound assay (migration) and anti-apoptosis assay (survival). The HUVEC proliferation and migration graphs **(Figure 6-4, Figure 6-5)** both show a significantly augmented effect in the presence of hASC-CM released from the composite on day 4 in comparison to the control group. The ability of the released CM to prevent HUVEC apoptosis was also studied as illustrated in **(Figure 6-6)**. In contrast to the control, the percent apoptosis in the case of HUVECs seeded with supernatant containing hASC-CM released from the matrix on day 4 was drastically reduced. The enhanced angiogenic effect of the CM is attributed to the ability of the delivery system to shield the encapsulated stem cell derived protein cocktail from enzymatic degradation. To demonstrate the individual effect of hVEGF and hbFGF, present in the hASC-CM, antibodies against the proteins were added. There was a marked decrease in HUVEC proliferation and migration and a sharp increase in apoptosis when anti-hVEGF was added to the hASC-CM highlighting the intense angiogenic effect of this protein on HUVEC growth. The effect of anti-hbFGF antibody added to the CM was not as severe as the absence of hVEGF but this could presumably be due to the lower concentration of hbFGF in the CM in comparison to hVEGF concentration. Thus through these bioactivity and inhibition studies by neutralizing antibodies, the overall effect of CM as well as individual effect of the two proteins considered was clearly observed and the advantage of delivering a cocktail of proteins rather than individual proteins was highlighted.

6.7 Conclusion

Our work presents for the first time the strategy of using a continuous delivery system loaded with stem cell derived growth factor cocktail for potential application in wound healing and tissue regeneration therapy. As evident from our results, the use of the PLGA MP/Coll composite system as a delivery vehicle for the paracrine factors or GF cocktail secreted by human adipose

derived stem cells, which not only protects but also allows sustained protein release, could be a better alternative to the direct injection of stem cells or bolus administration of individual growth factors which pose several complications and require multiple doses to obtain a significant regenerative response.

6.8 Acknowledgement

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6.9 List of Figures and Tables

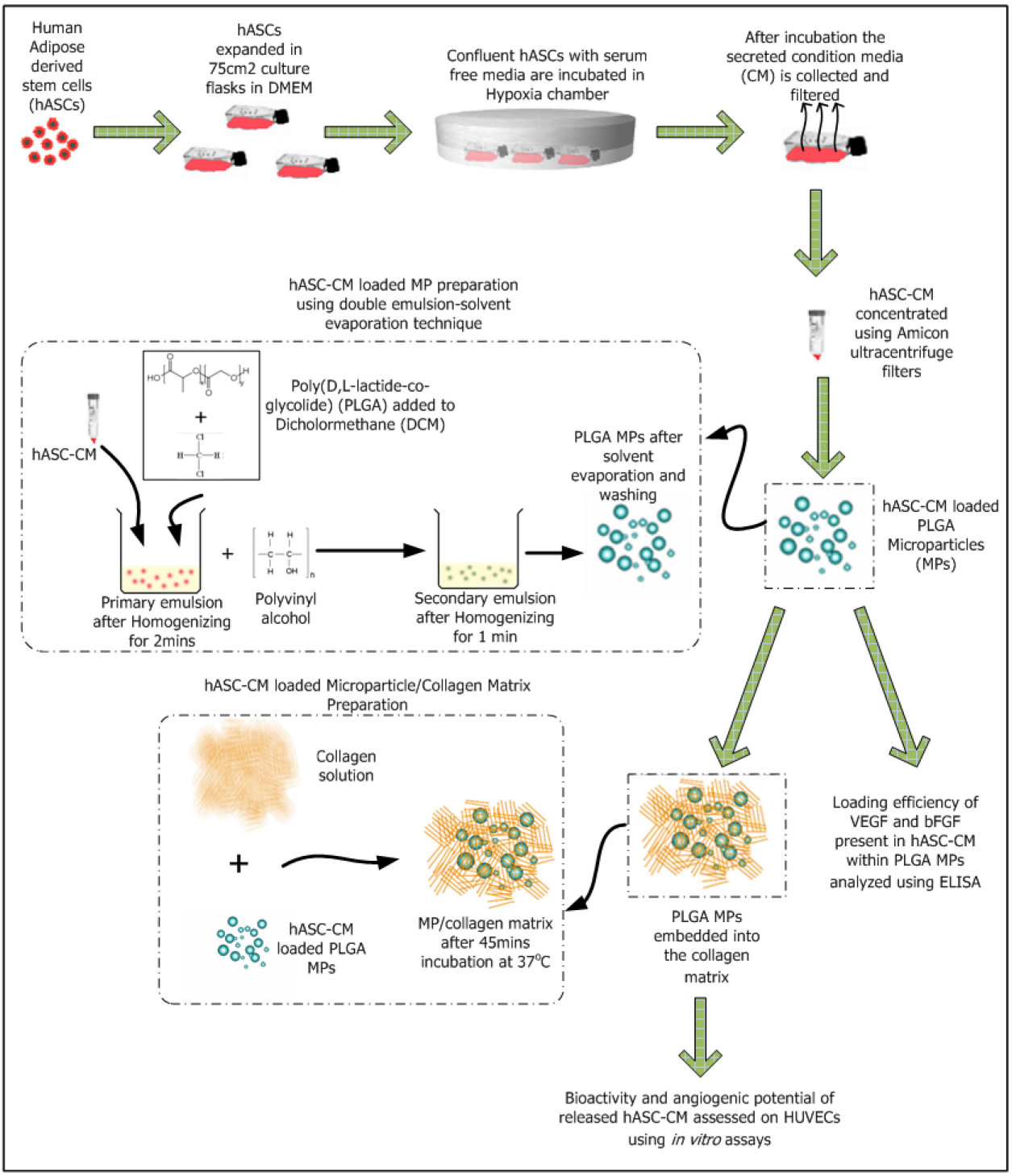

Figure 6-1: Schematic illustration of the entire procedure of PLGA MP/coll matrix development, starting with CM generation from adipose derived stem cells under hypoxic conditions followed by PLGA MP preparation and finally formation of the MP/coll matrix.

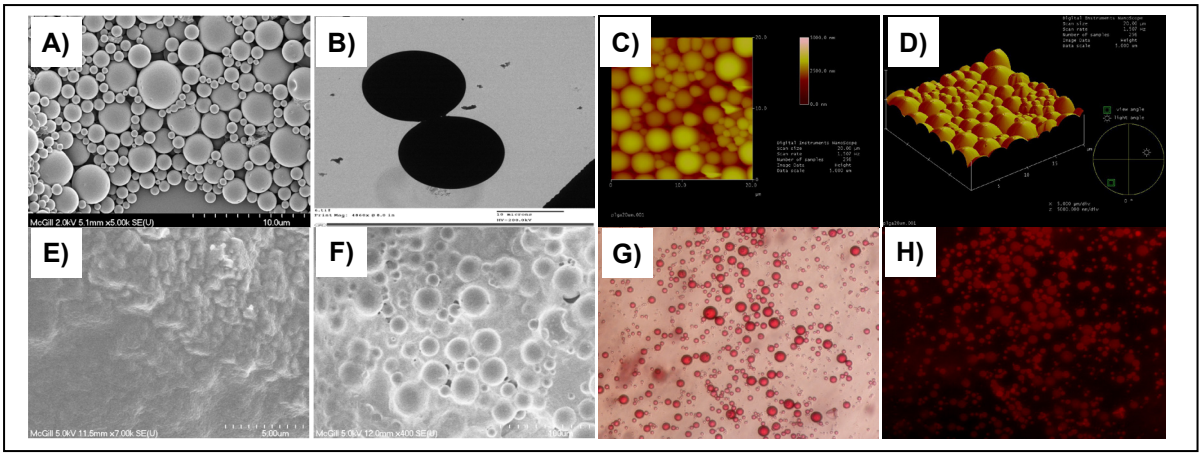

Figure 6-2: Microscope images of PLGA MPs

(A) SEM **(B)** TEM and **(C, D)** AFM images of PLGA MPs showed that the hASC-CM loaded MPs were perfectly spherical in shape with an intact surface devoid of wrinkles or cracks. SEM images of collagen matrix **(E)** without MPs illustrates the surface morphology of the collagen matrix without PLGA MPs and **(F)** with MPs showed that the PLGA MPs formed an integral part of the collagen matrix with no significant effect on MP morphology after incorporation. The incorporated PLGA MPs were observed to be uniformly distributed throughout the collagen matrix. **(G, H)** Images of the collagen matrix embedded with nile red loaded PLGA MPs taken using a fluorescent microscope under bright field and fluorescent field, 30X magnification. Scale Bar: 10µm **(A)**, 10µm **(B)**, 5µm **(C, D)**, 5µm **(E)**, 100µm **(F)**.

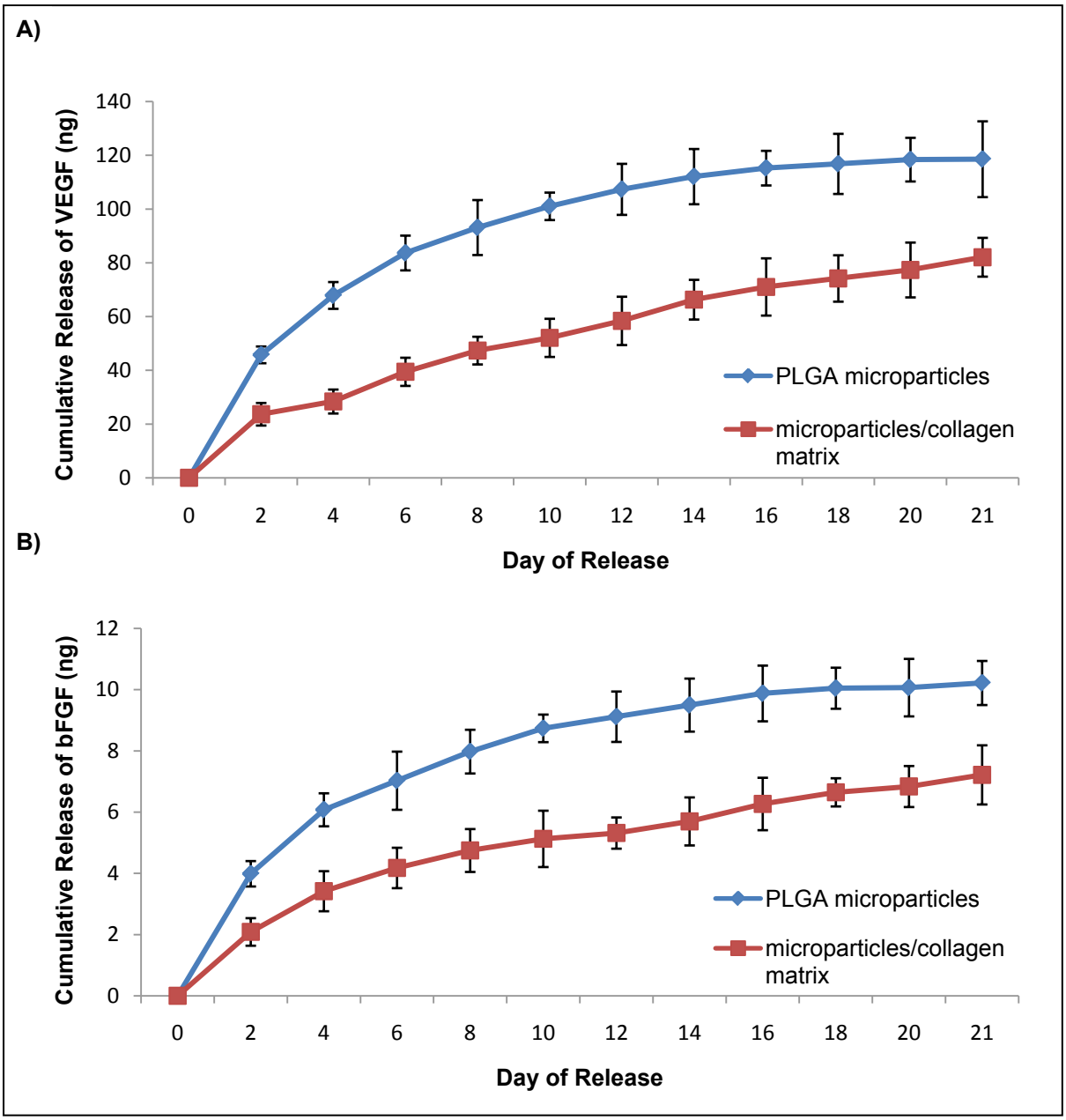

Figure 6-3: The *in vitro* release profiles of (A) hVEGF and (B) hbFGF present in hASC-CM released from only PLGA MPs (blue) and from the MP/coll composite (red) were investigated for 21 days.

The protein release curve was characterized by two phases: an initial burst phase followed by a slower constant release over time. Data, which is expressed as cumulative ng of either hVEGF or hbFGF released versus time of release, is the mean ± Standard Deviation of three replicates determined using ELISA.

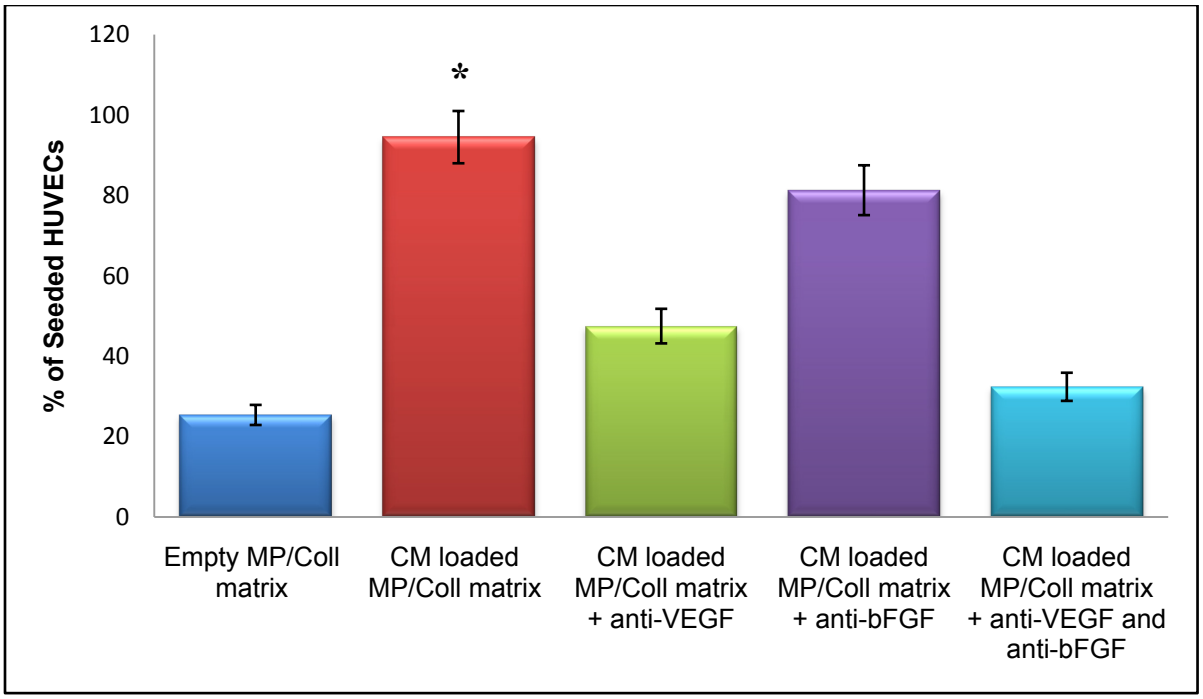

Figure 6-4: Effect of hASC CM released on day 4 from the MP/coll matrix on HUVEC proliferation.

The cells were incubated with 0.2 ml of the 50% fresh ECM (supplemented with 5% (v/v) FBS without cell growth supplements) and 50% of the supernatant from CM loaded MP/coll matrix (incubated with 2 ml serum free ECM) from day 4, and CM released from the MP/coll matrix on day 4 with 10µg/mL anti-hVEGF antibody and 5µg/mL anti-hbFGF antibody. Control wells received 0.2 ml of the 50% fresh ECM supplemented with 5% (v/v) FBS without cell growth supplements and 50% of supernatant when the empty MP/coll matrix placed in serum free ECM. Briefly, after 96 hours of incubation, data is determined as the mean percentage of endothelial cells on day 4 versus initial cell number (2×10^4cells/well) ± Standard deviation of three independent experiments. Results show that cells placed with supernatant from CM loaded MP/coll matrix had a significantly enhanced growth compared to the other groups (* $p < 0.05$).

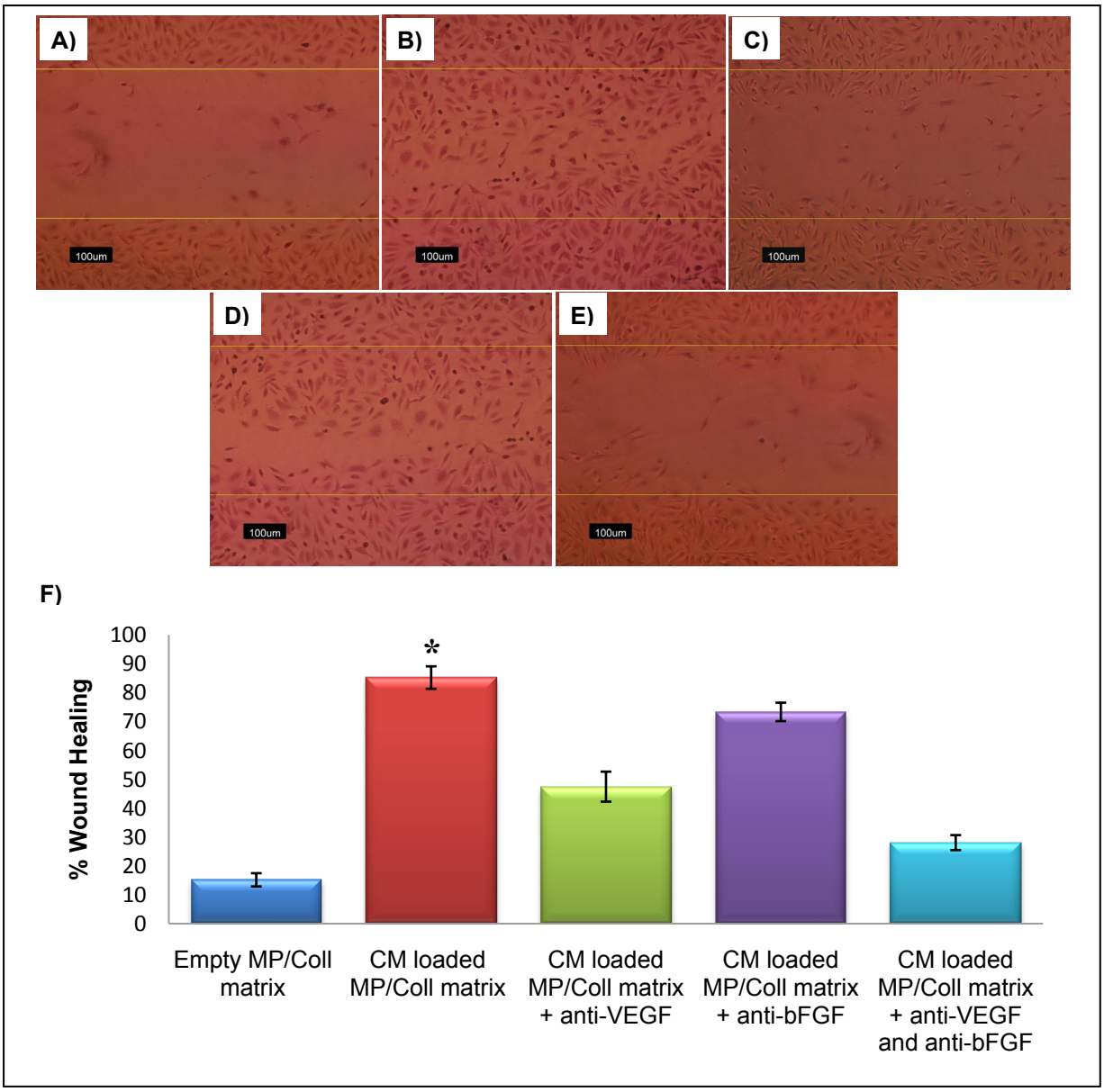

Figure 6-5: The ability of the hASC-CM released from the MP/coll matrix to assist HUVEC movement was demonstrated using a scratch wound assay.

(A-E) After cells reached confluence, they were serum starved, and a wound was created. The cells were incubated with 50% fresh ECM supplemented with 5% (v/v) FBS without cell growth supplements and 50% of the supernatant from CM loaded MP/coll matrix (incubated with 2 ml serum free ECM) from day 4, and released CM with 10µg/mL anti-hVEGF and 5µg/mL anti-hbFGF antibodies. Control wells received 50% fresh ECM (supplemented with 5% (v/v) FBS without cell growth supplements) and 50% of supernatant when the empty MP/coll matrix placed in serum free ECM. Representative fields were photographed under 20X magnification using light microscopy. **(F)** The number of cells that had moved to the wound area (between the yellow lines in images) were counted and illustrated in the graph. Data is representative mean ± Standard Deviation of 3 independent experiments. (*$p < 0.05$).

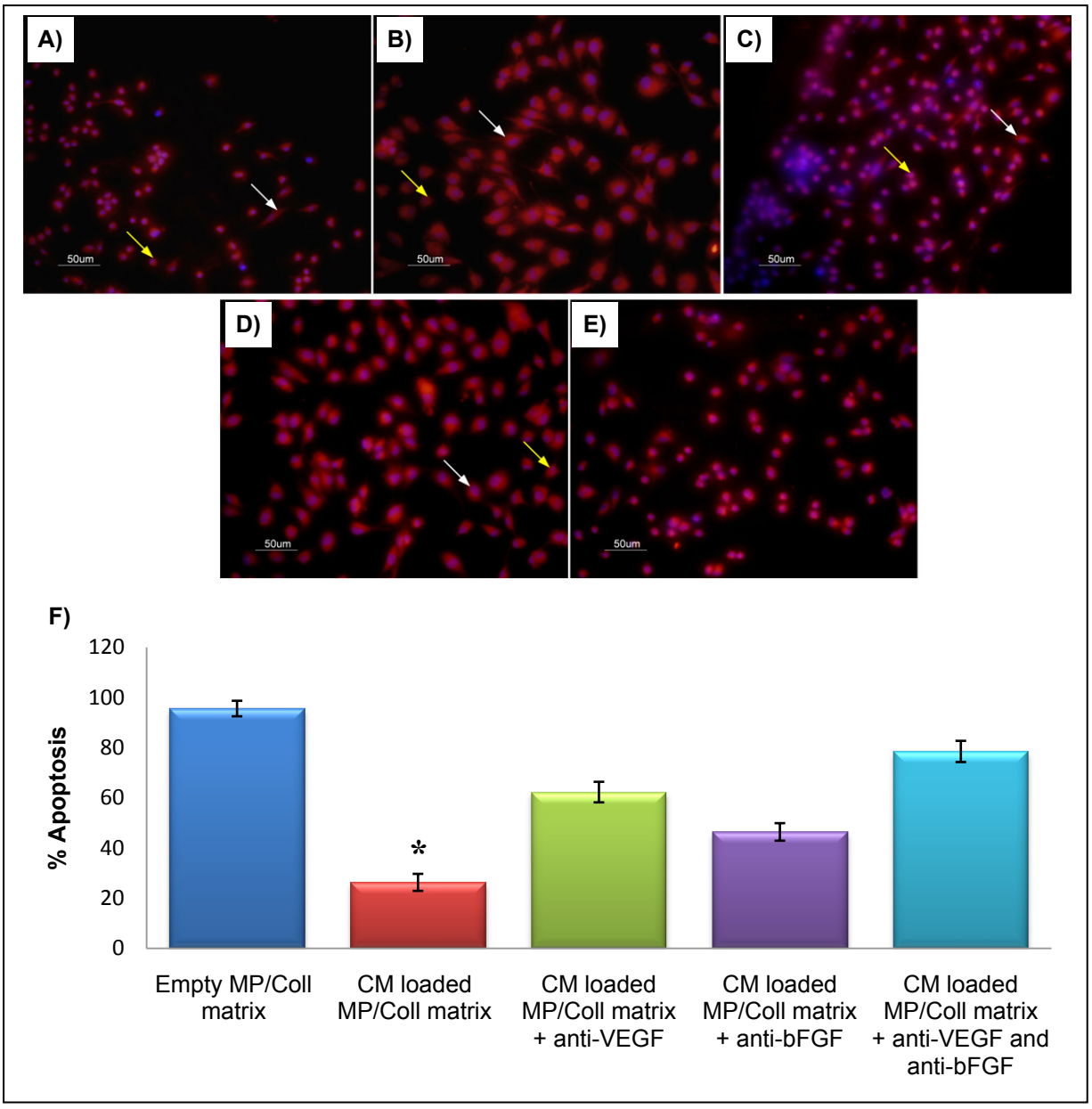

Figure 6-6: Fluorescent microscope images showing anti-apoptotic effect of supernatant containing hASC-CM on HUVECs for day 4.

(A-E) It was observed that on day 4 the protein cocktail released from the MP/collagen matrix into the supernatant significantly decreased HUVEC apoptosis compared to the control. A distinct change in the morphology of the cells undergoing apoptosis (indicated by yellow arrows) was observed in comparison to live cells (indicated by white arrows). **(F)** The anti-apoptotic effect of the hASC-CM released into the supernatant on the seeded HUVECs is demonstrated in a graph which illustrates the significantly lower apoptosis of HUVECs in comparison to the other groups (* $p < 0.05$). Three independent experiments were performed and the mean ± Standard Deviation is represented.

7 General Discussion

Today in Canada, cardiovascular diseases, which cause a gradual drop in the heart muscle cells and ultimately heart failure, are one of the leading causes of mortality[1]. Although significant advancement has been made in various surgical procedures and drug strategies, these methods still do not offer complete treatment options to heart disease patients and organ transplant still remains the only option for end-stage patients. Furthermore, with the issues of limited availability and organ rejection after transplant, numerous groups are now investigating alternative strategies such as gene therapy [169], protein therapy [156] and cellular therapy [170] i.e. the use of adult stem cells, with the aim to repair and regenerate the weakened myocardium.

Although several studies have demonstrated the potential and possibility of administering stem cells to facilitate tissue repair in regenerative medicine, there has been uncertainty over the mechanisms responsible for this regenerative effect produced by the stem cells after injection. Current hypothesis is that a paracrine effect created by cytokines or growth factors secreted by the injected stem cells is actually the key mediator and several studies have provided significant evidence for this, both *in vitro* and in small and large animal models[9, 56]. On the other hand, many groups have also demonstrated that the bolus injection of growth factors, although showing initial success, does not have a prolonged effect enough to promote tissue regenerative due to protein denaturation in the harsh *in-vivo* environment [18]. Thus an alternative approach is the use of controlled delivery systems such as microparticles and nanoparticles which not only provide a prolonged release but also protects the encapsulated growth factors from the harsh *in vivo* conditions. With this goal, we set forth to investigate the use of a polymeric controlled delivery particle system loaded with stem cell generated growth factor cocktail which could augment the therapeutic benefits of using the stem cell derived growth factor cocktail for possible application in cardiac remodeling and tissue repair therapy.

First, in order to understand the mitotic and anti-apoptotic effect as well as observe the release kinetics of a combination of proteins using a controlled delivery system, our initial study involved co-encapsulation of the pro-angiogenic proteins hVEGF and hAng-1 in albumin NPs. Although studies have shown the ability of hVEGF to stimulate angiogenesis [107-114], there is still uncertainty as to whether the presence of this protein alone would suffice in the achievement of functional and mature vessels with evidence indicating that hVEGF could in fact cause

pathological and immature vessel formation [45, 115, 116]. Other studies have demonstrated that angiopoietin-1 has the ability to stabilize and assist in the maturation of blood vessels [117-119], and in fact abrogates endothelial cell apoptosis [120, 121]. It was hypothesized that delivery of a combination of these two proteins could provide promising therapeutic advantages. Our results showed that application of a NP system enabled efficient and controlled release of the proteins over a certain period of time. An optimal concentration of glutaraldehyde was chosen for coating the NPs with tolerable toxicity which provided efficient and controlled release of the co-encapsulated proteins. Particle characterization studies showed that the size of the NPs cross-linked with glutaraldehyde varied between 101.0± 0.9 nm and the zeta potential was found to be -18 ± 2.9 mV. These results were observed to be in accordance with previously published results [141]. The NPs were incubated for two weeks to determine the release profiles of the proteins. At the end of the two week incubation period, it was observed that 49 ± 1.3% of hAng-1 and 59 ± 2.1% of hVEGF had been released from the NPs. The effect of the released proteins on endothelial proliferation and apoptosis was also observed to determine the mitotic and anti-apoptotic ability. The results indicate that the released proteins were biologically active and the combined application of both the proteins demonstrated a significantly high proliferatory and anti-apoptotic effect on HUVECs as compared to the effect demonstrated by the released individual proteins. Thus through this study, not only was the advantage of sending a combination of proteins instead of single proteins highlighted but also the benefit of using a controlled delivery system instead of single protein injections which fail to provide a prolonged therapeutic effect. These studies helped understand the advantage of using a combination of GFs instead of single proteins released from a protective controlled release system which highlighted the advantage of our further investigations using the stem cell derived protein cocktail released containing multiple growth factors from controlled delivery particles.

Once we observed the augmented pro-angiogenic effect of using a combination of proteins slowly released from albumin nanoparticles on endothelial cells, our next set of experiments were done to assess the possibility of encapsulating stem cell derived growth factors into a polymeric controlled delivery system for protected and sustained release. PLGA was chosen as the polymer to load the stem cell derived growth factor cocktail as there have been several studies demonstrating the biocompatibility, biocompatibility and safety of use of this polymer in addition to being approved by the FDA [91]. As demonstrated in previous work, the stem cell-

generated growth factor cocktail contains a large spectrum of cytokines [56, 155]. Furthermore, studies have also highlighted the fact that generation of the stem cell CM under hypoxic conditions augments several key pro-angiogenic factors, such as hVEGF and hbFGF, and we implemented this strategy to harness the factor cocktail from stem cells in our studies[161]. However in our research we did not investigate the role of each cytokine present in the cocktail and emphasized on a few key pro-angiogenic proteins which promote cardiac regeneration using stem cell therapy [159]. The quantitative amount of hVEGF in the CM secreted from the stem cells after incubation for 16 hrs with serum free medium under hypoxic conditions was determined using ELISA. We choose to identify this protein present in the stem cell derived cocktail as it is known to be an essential pro-angiogenic stimulant for cardiac tissue repair [9]. Furthermore, studies have shown that hVEGF is present in augmented amounts in the myocardium post infarct after administration of stem cells [167]. The concentrated stem cell factor cocktail loaded PLGA particles were developed and particle size was characterized and was found to be in the range of 100-400 nm as previously demonstrated. *In vitro* release studies showed that the GF cocktail was released at a controlled and sustained rate for over a 21 day period [148]. The functional (mitotic and chemotactic) activity studies using the released factor cocktail on HUVEC proliferation, migration and tube formation assays demonstrated retention of bioactivity of the released factors and highlighted the advantage of co-encapsulating BSA during the particle formation, known to effectively shield the encapsulated biomolecule from the particle surface and prevent protein deactivation during particle preparation [93].

In-vivo studies using the polymeric particles were done in an infarct rat heart model to assess the possible potential of application in tissue regeneration and treatment therapy. *In vivo* studies using the growth factor cocktail loaded PLGA NPs demonstrated a functional improvement in the infarcted rat heart model observed through the echocardiographic data, reduced infarct size, a thicker left ventricular wall and increased angiogenesis and arteriogenesis. Animals in the group treated with growth factor loaded PLGA NPs showed significantly greater improvement than those receiving the free growth factor cocktail or empty NPs which provided evidence of the enhanced therapeutic effectiveness of our system. Forrmiga et al. recently demonstrated through pre-clinical trials that encapsulation of VEGF into PLGA MPs led to an improved cardiac function as compared to free VEGF protein[18]. Our augmented results highlight the benefit of

delivering a combination of stem cell derived growth factors in contrast to individual proteins for potential application in cardiac tissue regeneration therapy.

The previous set of experiments were done as a proof of concept to first assess the potential of using polymeric particles as delivery vehicles for stem cell derived growth factors to the target site with the goal of mimicking the paracrine effect created by injected stem cells. In the next set of experiments, the aim was to develop a composition that could provide an even more extended and protected release of the encapsulated factors. Moreover, PLGA particles are known to be susceptible to phagocytosis by macrophages and do not remain at the injected site for too long. Furthermore, several groups have highlighted that bone marrow derived stem cells are difficult to obtain and grow in large numbers and the use of a suitable easily obtainable alternative stem cell type would greatly increase the potential clinical application of this tissue regeneration strategy. Thus, human adipose derived stem cells which are easily accessible and require only a simple minimially invasive procedure for harvest make a promising alternative to the use of bone marrow derived stem cells and have been chosen in this study[171]. We developed polymeric MPs loaded with adipose derived stem cell growth factor cocktail which were then embedded into a hydrogel matrix. As demonstrated by Borselli et al., collagen has excellent properties of biocompatibility and low antigenicity and has widely been used in matrix development for applications in regenerative therapy [93]. The hASC-CM loaded PLGA MPs developed in our study were incorporated onto a collagen matrix during fabrication and it was observed that uniformly distributed PLGA microspheres formed an integral part of the collagen matrix without deformations in the MP structure. Release of hVEGF and hbFGF from the MP/collagen matrix was observed over a period of three weeks. Furthermore, to evaluate the angiogenic potential of the hASC-CM released from the MP/collagen composite, we used *in vitro* angiogenic assays. The results show a significantly augmented effect in the presence of hASC-CM released from the composite on day 4 in comparison to the control group. The enhanced angiogenic effect of the CM is attributed to the ability of the delivery system to shield the encapsulated stem cell derived protein cocktail from enzymatic degradation. This study proposes a novel method of delivering GFs secreted by stem cells using a polymeric membrane system with the goal of mimicking the release of GFs from implanted cells.

This strategy of delivery provides the advantage of protected, prolonged and continuous release of GFs instead of bolus administration, shown to require multiple injections to achieve acceptable results. It is hypothesized that with further studies, this strategy could completely replace the administration of stem cells in regenerative therapy and the drawbacks related to their use.

8 Summary of Observations

In this thesis research, the initial study highlighted the benefits of delivering a dual combination of growth factors using delivery particles to promote angiogenesis. Then, the novel strategy of using polymeric controlled release polymeric particles for delivery of the multiple growth factor cocktail harnessed from stem cells was investigated. This study involved the preparation and characterization of controlled release PLGA particles loaded with stem cell factors followed by assessment in promoting angiogenesis in an *in vivo* myocardial infarct rat model. Lastly, a collagen matrix embedded with the PLGA particles was prepared and characterized to obtain a more prolonged release of the encapsulated stem cell factors.

The following is a summary of the observations and results:

1) **Preparation and characterization of dual protein loaded HSA NPs**

   The benefit of using dual proteins released from delivery particles to promote angiogenesis was highlighted through the use of HSA NPs coencapsulating two proteins, hVEGF and hAng-1. Using the previously established coacervation method according to optimized conditions, we were able to manufacture negatively charged HSA NPs of size between 101.0 ± 0.9 nm. Particle characterization studies were done and results show that stable non toxic particles suitable for protein delivery were formed at a concentration of 40µl of 5% (w/v) glutaraldehyde.

2) **Protein encapsulation efficiency and release kinetics from HSA NPs**

   The encapsulation efficiency of both the proteins was determined and results illustrated that 49% and 55% of hAng-1 and hVEGF was loaded inside the HSA NPs. A three week protein release study was then performed, during which the release profile of the two proteins was observed. Results show a burst release of both the proteins within the initial two days followed by a lower steady release phase

3) **Functional activity studies and mitotic and anti-apoptotic effect of released protein on HUVECs**

   In vitro functional angiogenic assays: proliferation and anti-apoptosis assays with endothelial cells were performed to confirm that the released proteins remained bioactive

even after the particle development procedure. Our results showed that the highest proliferation (89.62%) and the lowest percent apoptosis (16.44% to 10.42% for day 2 to 14) was observed when HUVECs were seeded with supernatant containing both the released proteins in comparison to the individual protein effect, highlighting the advantage of delivering a combination of growth factors. These results also showed the ability of the delivery particles to protect the encapsulated proteins from degradation.

4) **Preparation and characterization of concentrated stem cell factor loaded PLGA NPs**

Several studies demonstrate the potential of direct administration of the stem cell derived growth factors towards wound healing and tissue repair. Through our results, we show that the use of controlled delivery particles could augment this potential. PLGA NPs loaded with stem cell derived growth factors were obtained using double emulsion solvent evaporation technique with the average size of the NPs ranging between 100 and 400 nm. Particle characterization studies were performed and results illustrate that stable and spherically shaped stem cell factor loaded PLGA NPs were formed.

5) **Encapsulation efficiency and release kinetics from PLGA NPs**

The encapsulation efficiency of hVEGF present in the loaded factor cocktail was determined using the hVEGF ELISA assay and results show the efficiency to be 48.2 ± 2.5%. Our results from the three week protein release profile study demonstrate that hVEGF release present in the growth factor cocktail was characterized by two phases: an initial burst phase followed by a slower sustained release over time.

6) **Biofunctionality assessment of stem cell conditioned medium released from NPs**

Angiogenic assays: proliferation, migration and tube formation assays using endothelial cells were used to examine if the released factor cocktail remained functionally active even after particle preparation procedure. Our results show that a significantly augmented effect was produced when HUVECs were seeded with supernatant containing stem cell factors released from the PLGA NPs in contrast to the controls which confirmed that the factors retained their biofunctionality.

7) ***In vivo*** **analysis of the developed stem cell factor cocktail loaded PLGA NPs**

An *in vivo* myocardial infarct rat model was used to examine the potential of the developed stem cell factor cocktail loaded PLGA NPs for possible heart tissue repair applications. Our results demonstrate a significant improvement in cardiac functionality in the animals treated with stem cell growth factor loaded PLGA NPs in comparison to the animals receiving free factors or empty NPs. Similarly, an increased capillary and artery density was measured in the group receiving stem cell growth factor loaded NPs.

8) **Preparation and characterization of PLGA MP embedded hydrogel composite**

A delivery system allowing a more localized and extended release of the stem cell harnessed factor cocktail was developed in the final study. The stem cell growth factor cocktail was harnessed from the easily accessible adipose derived stem cells instead of bone marrow derived stem cells and loaded into PLGA MPs. These MPs were then embedded into a collagen hydrogel to ensure a more localized and extended protein release. Results from our particle characterization studies demonstrated that the composite system so formed was stable, with particles devoid of wrinkles or cracks.

9) **Protein release profile from composite**

Release profiles of hVEGF and hbFGF present in the factor cocktail released from the composite system was then determined over a three week period. Our results show an extended and prolonged release of the proteins from the composite system as compared to just PLGA MPs.

10) **Biofunctionality assessment of stem cell derived growth factors released from composite**

The ability of the released stem cell derived factor cocktail to stimulate proliferation, promote wound healing and prevent cell apoptosis was observed using endothelial cell angiogenic assays. Our results demonstrate a significantly improved effect created by the released factor cocktail as compared to the control groups which highlighted the retained bioactivity and angiogenic ability of the released cocktail from the composite system.

9 Conclusions

Although the ability of stem cells to facilitate tissue repair has been demonstrated in several studies, uncertainty over the mechanisms actually responsible for this regenerative effect has recently arisen. A new hypothesis is that a paracrine effect, created by growth factors secreted by the injected stem cells, is actually the key mediator. The main goal of this study was to investigate a novel method of using biocompatible controlled delivery particle systems loaded with stem cell harnessed GFs for potential application in cardiac remodeling and repair, providing protection as well as a sustained and prolonged release of the encapsulated factors.

In the first study, we coencapsulated two pro-angiogenic recombinant proteins freely available in the market, hVEGF and hAng-1, to understand the release profile of the proteins from the delivery particles and assess the effect on functional activity of the proteins after release. We were successfully able to demonstrate the favorable mitotic and anti-apoptotic effect of the two released proteins on endothelial cells as well observe the protein release profiles from the albumin NPs. These functional activity studies showed that the encapsulated proteins remained functional even after the particle preparation procedure. Through this study, the advantage of using a combination of proteins instead of individual proteins encapsulated inside a polymeric matrix was highlighted. In the second study, we then move to the main portion of the project, where bone marrow derived stem cell derived growth factors obtained under hypoxic conditions were loaded inside PLGA NPs. Surface characterization and release kinetics studies were performed followed by functional activity studies on endothelial cells using angiogenic assays which demonstrated the potential of this system in tissue regeneration therapy. A pre-clinical MI rat heart model was used to assess the possibility of using PLGA NPs loaded with stem cell derived growth factors in damaged cardiac tissue regeneration. In this study, the advantages of using a delivery system for continuous delivery of the loaded growth factor cocktail instead of free CM from the stem cells is highlighted. Not only an angiogenic but also an arteriogenic effect was observed leading to improved cardiac functionality after MI. Our system not only provided better results compared to free protein administration but could remove the need for direct injection of stem cells which has several drawbacks as mentioned before. In the final chapter, with the aim of creating a system that allows an extended release of growth factors, stem cell factor cocktail loaded PLGA particles were embedded into a collagen matrix. Furthermore, the

use of bone marrow derived stem cells was replaced with the more easily accessible stem cells from fat tissue. Such a composition could be used in several tissue regenerative and wound healing applications. In conclusion, the results from this study bring forth the advantages and benefits of using a delivery system for controlled release of the stem cell derived growth factors with potential application in tissue regenerative therapy, which could one day completely evade the concept of direct administration of bolus proteins or stem cells. With further studies, such a strategy could be up scaled with future pre-clinical and clinical research in patients.

10 Recommendations and Future Applications

This study provides a novel strategy for the administration of stem cell derived growth factors, shown in previous studies as possibly being the main mechanisms behind the regenerative effect created by stem cells. The stem cell harnessed growth factors were encapsulated inside a delivery system allowing controlled and sustained release along with protein protection to enable prolonged availability of the growth factors at the damaged site. With further research and improvements this technique could form the basis of future clinical trials in patients.

1) Understanding the role played by each cytokine in the cocktail on tissue regeneration is also needed. This could allow the possible application of genetically modified stem cells to alter the secretion of key pro-angiogenic factors into the CM, needed in augmented amounts, for *in vivo* tissue regeneration.
2) The potential of this therapeutic system can be further improved if the release profile of the other cytokines present in the stem cell cocktail from the PLGA nanoparticles is also observed.
3) Further studies on deciding an optimal timing of administration of the NPs also needs to be done to promote improved tissue regenerative effects.
4) An *in vivo* biodistribution study should be performed to check for the possibility of NP aggregate formation and embolization.
5) PLGA has been selected as the polymer of interest in this study. However, other biocompatible polymers should be explored depending on the tissue to the regenerated such that the rate of degradation of the polymer is closely proportional to the rate of regeneration allowing a prolonged release of the growth factors at the target site.
6) *In vitro* characterization studies of the MP/Coll matrix have been done in this study, however *in vivo* studies using this composite should be done to assess the potential of this system in wound healing and tissue regeneration.
7) Targeted delivery of the stem cell derived growth factor cocktail loaded polymeric delivery systems could be possible by surface conjugation with antibodies against cell – adhesion molecules.
8) The polymeric delivery particles could be surface modified with a biopolymer to invoke interaction with fibronectin, found in excess at wound healing sites.

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