IMPROVING STEM CELL SURVIVAL AND DIFFERENTIATION IN ISCHEMIC
AND INFLAMMATORY TISSUES

DISSERTATION

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ABSTRACT

Ischemia disease, mainly including heart ischemia, brain ischemia, and limb ischemia, are severe vascular diseases. Millions of people worldwide are suffered from these diseases and the death rate has remained high. Ischemia is induced when blood supply to the tissue is not enough due to the blockage of blood vessel. Cells die and tissues are damaged because of the ischemic environment. To regenerate the damaged tissue and restore lost tissue function, stem cell therapy is regarded as a promising approach. Stem cells are multipotent cells which can renew themselves and also may differentiate into many cell types in the host tissue. However, efficacy of the therapy is extremely low when the stem cells are implanted directly into the target tissue area. Dramatic cell death is caused by three major factors: no proper stem cell carrier exists as an ECM and delivery vehicle, harsh ischemic conditions (low oxygen and low nutrient), and immunorejection and inflammation.

In this thesis, the above issues were addressed accordingly in order to improve the implanted cell survival. A series of polyNIPAAm based biodegradable thermosensitive hydrogels were synthesized to serve as proper carriers of stem cell, biomolecules (like growth factors), and oxygen releasing microspheres. In Chapter 2, growth factor bFGF was encapsulated in hydrogel together with the cells. The bFGF could sustain release from the hydrogel for 28 days and remain bioactive. The released bFGF was able to enhance
stem cell survival under ischemic conditions *in vitro* and *in vivo*. This system also promoted angiogenesis to restore blood perfusion *in vivo*.

To overcome the low oxygen environment, a novel oxygen releasing microspheres were fabricated by electrospray technique. The core shell structured microspheres were based on PLGA for the shell and H₂O₂/PVP complex for the core. The complex is released when PLGA is degraded, and generated oxygen via catalase. The complex-stabilized H₂O₂ and the slow degradation of PLGA guaranteed a sustained release for a relatively long period of time. The oxygen release system could enhance stem cell survival and proliferation *in vitro* and *in vivo*. To make the oxygen releasing microspheres more functional and could perform environment responsive releasing behavior, several upgrade version of microspheres were developed as described in *Chapter 3*, *Chapter 4*, and *Chapter 5*. Catalase was conjugated on the surface of the microspheres to make the oxygen releasing system more accessible. By introducing fluorescent agent hepericin into the complex, imagable oxygen releasing microspheres were fabricated and could be fluorescently detected both *in vitro* and *in vivo*. In addition, hypoxia–sensitive degradable polymer was synthesized as shell material. The releasing kinetics of the oxygen releasing microspheres were shown to be environmentally responsive to the oxygen level. All the above mentioned advanced oxygen releasing system could promote stem cell survival under ischemic conditions.

Necrosis of the damaged tissue under ischemic conditions recruit large immune cells and protein which secrete pro-inflammatory cytokines. The small cytokines, mainly TNF-α and IL-1β, could penetrate into the hydrogel and stimulate cell apoptosis. Peptides
which showed binding affinity to specific cytokines were used to modify the hydrogel surface via biotin-avidin interaction. The newly developed hydrogels were able to block the cytokines and eliminate inflammation. The survival and differentiation of the encapsulated stem cells were significantly enhanced and promoted in vitro and in vivo.

The developed strategies and approaches to improve stem cells survival under ischemic and inflammatory conditions should offer therapeutic options for tissue regeneration.
Dedicated to my parents

Jian Li

and

Xueqin Li.
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CHAPTER 1: Introduction: Approach to improve cell survival in stem cell therapy for treating ischemia induced disease

1.1 Introduction

Heart disease, especially myocardial infarction (MI), has a leading morbidity and mortality worldwide [1]. MI is a major cardiovascular disease which causes massive cell death and partial loss of heart function. When MI occurs, because of no blood supply, an ischemic environment (low oxygen and low nutrient) forms in the infarct region which lead to heart tissue death. The necrotic tissue is then removed by macrophages and eventually scar tissue is formed. Furthermore, the left ventricular wall of infarct heart becomes thinner and the left ventricular chamber dilates. Because cardiomyocytes are unable to regenerate, heart function cannot be restored. In the end, the process leads to cardiac dysfunction and eventually heart failure.

Heart transplantation is a current method addressing this issue but extremely limited by the number of donor organs and long-term immune problems. Development of a therapeutic method to repair the damaged heart tissue is a desirable strategy. In such conditions, stem cell therapy is pursued as a potential approach to regenerate the damaged heart tissue and promote angiogenesis. By injection of stem cells into the infarct region, the stem cells engraft in the damaged tissue area and differentiate into cardiomyocytes to
replace the lost cardiomyocytes. The tissue is regenerated and heart function is restored afterward.

However, it is reported that when injecting cells into heart directly, approximately 90% of cells were lost to the circulation, leaked, or squeezed out of the injection site because of the heart’s motion [2]. For the cells retained in the injection site, most of the cells died within the first few weeks and only a small percentage of survived cells are observed to be differentiated [3]. Thus, the efficacy of direct stem cell injection method is extremely low and clinical application is limited. The causes of such a situation are mainly two aspects: cell deliver system and ischemic environment. The commonly used saline solution has very low viscosity and cannot hold the cells in the injection site. In the infarct heart, an extremely low oxygen and low nutrient ischemic environment is generated and the transplanted cells struggle to survive in such harsh conditions.

Ischemic-induced brain injury, mainly referring to stroke and traumatic brain injury (TBI), causes a large number of death and disability globally [4, 5]. Even though a relatively high percent of patients can survive following a stroke, stroke-induced brain damage results in disability to the patient because of the neural cells dysfunction in the brain [6].

When stroke occurs, there is a disturbance in the blood supply to the brain which is caused by ischemia or hemorrhage. Ischemic stroke occurs because the blood vessels of the brain are blocked by thrombosis or arterial embolism, or by cerebral hypoperfusion – ultimately, the blood supply is interrupted. Hemorrhagic stroke occurs because ruptured blood vessels bleed into the brain parenchyma or the subarachnoid space around the tissue,
or the structure of the vasculature is abnormal. Most incidences of stroke are due to acute ischemic stroke accompanied by hemorrhaging take place inside the ischemic region. It was reported that there is a connection between an acute myocardial infarction and hemorrhagic stroke following afterward. Even though this complication is rare, results are quite serious [7].

Thrombolytic method is currently a widely used option to treat acute ischemic stroke. Recombinant tissue plasminogen activator (t-PA) is used in this therapy, and is approved by FDA. However, the treatment time window is limited for the thrombolytic treatment to perform on the patient to resume the blood flow in brain [8]. In addition, there is evidence that showed some patient may be in a risk of hemorrhaging after t-PA treatment. Stem cell therapy is therefore considered to be an alternative new method that showed encouraging results [9]. The transplanted pluripotent stem cells, such as neural stem cells (NSCs), can self-renew, proliferate and differentiate to promote neurogenesis and nervous tissue regeneration, and brain function recovery. However, low retention and cell survival rate were reported due to the ischemic conditions and induced inflammatory response [10].

A proper carrier plays a key role to increase cell retention and survival rate after transplantation. In tissue engineering, biomaterials are widely used and investigated to address this. An injectable biomaterial can be used to deliver cells into the infarct heart through a syringe and needle. The injected biomaterials should help increase the cell retention, and at same time provide cells with a microenvironment viable for cell survival. In addition, they can also be used to support the infarct heart wall to avoid ventricular remodeling.
To address the issue of cell survival under ischemic conditions, many approaches were reported. Ischemic preconditioning cells improved ischemic tolerance of cells in the infarct heart tissue. Genetic modulation of cells prior to transplantation enhanced cell viability and apoptotic resistance by delivering growth factors and anti-apoptotic factor genes into the cells.

1.2 Stem cells for the therapy

After myocardial infarction and stroke, even though the heart and brain attempt to self-repair, cardiomyocytes and neurons cannot regenerate by adult host tissue. Stem cells have the ability to self-renew and potential to differentiate into cardiac cell types and central nervous system cell types. To replace the lost cardiomyocytes in heart and neurons in brain and regenerate new cells to restore the heart and brain functions, injection of stem cells is an attractive therapeutic approach which shows great potential in repairing hypoxic-ischemic induced damaged heart and brain. The feasibilities and behaviors of different types of stem cells in transplantation, proliferation, and differentiation were investigated. For example, induced pluripotent stem cells (iPSCs), mesenchymal stem cells (MSCs), neural stem cells (NSCs), embryonic stem cells (ESCs) and neuronal progenitor (NPCs) are commonly put into use and study.
1.2.1 Embryonic stem cells (ESCs)

Embryonic stem cells (ESCs) are pluripotent stem cells and have the ability to differentiate into any cell in the body. ESCs have the potential of unlimited proliferation and regeneration ability theoretically. In a MI stem cell therapy study, ESCs are proved to have the ability of differentiating into cardiomyocytes to replace the lost cells in the infarct tissue and restore the functions of the host heart \([11]\). Based on the properties of ESCs, the cardiomyocytes required to regenerate functional myocardium can be derived from ESCs via simple cell culture technique. In preclinical studies, ESCs were applied to the injured cardiac tissue and the result showed that the ESCs can differentiate and damaged tissue can be repaired \([12, 13]\). ESCs are also considered to be an ideal transplantation stem cell type for stroke induced brain damage repair. Many *in vitro* and *in vivo* studies on ESCs have proven that ESCs can differentiate into major cell types in brain \([14-16]\).

1.2.2 Mesenchymal stem cells (MSCs)

Mesenchymal stem cells are multipotent stem cells, which can differentiate into many cell types such as cardiomyocytes, vascular endothelial cells, and even neurons \([17]\). In addition, MSCs have the capacity of self-renewal and proliferation. Therefore, MSCs are extensively used in tissue regeneration, including that of ischemic heart and brain.

Toma et al. \([18]\) injected hMSCs into adult murine heart to study the differentiation of hMSCs. Despite only a small percentage of the injected hMSCs surviving, the immunohistochemistry result showed that the engrafted cells differentiated into cardiomyocytes. Miyahara et al. \([19]\) transplanted monolayered MSCs into infarct rat heart;
neovascularization was observed after 4 weeks as well as cardiomyocytes and undifferentiated cells. Furthermore, to study long-term effects of MSCs based therapy, Dai et al. [20] investigated MSCs transplanted into infarct heart in both short and long periods of time. Similar results were found after two weeks supporting MSCs can replace the dead cells, and cardiac markers (muscle and endothelium) showed differentiation. However, the differentiation process was not fully completed into an adult cardiac phenotype after 6 months.

To recover injured brain, Liu et al. [21] injected eMSCs which overexpress green fluorescence protein (GFP) into ischemic rat brains. Migration of eMSC to the infarct area was observed. And the engrafted MSCs were proven to differentiate into neurons and vascular endothelial cells. After 4 weeks, the infarct volume was reduced and brain function improved. Onda et al. [22] also found that hMSCs induced neuroprotection, angiogenesis, and improved the blood flow near the border of the ischemic region. Even though the MSCs, which are capable to differentiate into neurons, were proved to have promise for stem cell therapy application towards neurological disease, there was doubt from Franco et al. [23] whether the MSC differentiated neurons could function properly.

1.2.3 Neural stem cells (NSCs) and neural progenitor cells (NPCs)

Neural stem cells have the capacity of self-renewal and multipotency. NSCs can grow, proliferate, and differentiate both \textit{in vitro} and \textit{in vivo} [24]. Two kinds of neural stem cells, endogenous and exogenous, are used in stem cell therapy for brain repair. Endogenous neural stem cells are isolated from adult rodent central nervous system and can be cultured
Exogenous neural stem cells can be obtained from many tissue sources such as embryonic stem cells [27], bone marrow-derived mesenchymal stem cells [28], fetal stem cells [29] and adult nervous systems [26]. Bacigaluppi et al. [30] found that NSCs transplanted into stroke mice brain can still suppress inflammation and glial scar formation 3 days later. After 18 days, by providing neuroprotection, the function of stroke brain was improved, which indicates the time window can be extended. NSCs from subventricular zone were used for transplantation into stroke rat brain in one study of stroke brain [31]. Cells were labelled by superparamagnetic particles and magnetic resonance imaging (MRI) was used to investigate transplanted NSCs survival and migration. It was found that NSCs can also differentiate into neurons in vivo by detecting different neuronal markers [32]. In other studies [33-35], NSCs were also used to treat ischemic brain and the results showed migration and integration of NSCs to the injured tissue. New neuron generation, infarct size reduce, target tissue angiogenesis, and some function recovery were observed afterwards. NPCs can be isolated from fetal nervous system, embryonic brain, or directly derived from ESCs. NPCs have the ability to differentiate into neurons, glia, and oligodendrocytes in vitro and in vivo [36, 37]. NPCs which are derived from ESCs are optional for transplantation.

### 1.3 Biomaterials

Proper biomaterials matrices, when co-transplanted with the stem cells, play critical roles in stem cell therapy for the heart and brain tissue engineering. To meet with the basic requirements, the biomaterials must be biodegradable, biocompatible, biomimetic and can
protect the stem cells from attack of immune cells and proteins. To be specific, the biomaterials should have the property of controlled biodegradability, which means the degradation rate of the materials should coincide with the formation of new generated tissue. The degradation products should be bioresorbable and non-toxic to eliminate common inflammatory responses in a long period of time and allow the ingrowth of the tissue. The implanted biomaterials serve as carriers of the cells during transplantation and regeneration – therefore cytocompatibility is essential. The biomaterials should have good cells affinity so cells can adhere to the materials, live, and proliferate normally. In addition, the interaction between the materials and cells is enhanced. As the biomaterials are implanted into the host tissue, they should mimic the biological environment of the implantation site and retain similar properties to that of the surrounding host tissue, for example: stiffness. In addition, the materials should be chemically stable, which means the materials can preserve mechanical properties and maintain structures under physiological conditions after implantation into the body. For materials used in treating infarcted heart and brain tissue, additional requirements should be considered: firstly, the materials must be able to exhibit proper mechanical properties to support the infarcted heart wall after implantation and be also flexible to avoid heart remodeling after MI, and secondly the materials used to treat injured brain should be soft like the brain tissue.

The biomaterials that can be applied to therapeutic treatment usually fall into two major categories: natural materials and synthetic materials. Natural materials are biologically-derived materials which preserve inherent bioactivity and porous structure. Synthetic materials are designed and synthesized via chemical method to pursue desired properties
and functions. Usually the synthetic materials are able to be modified with peptides, growth factors, or functional molecules. In clinical stem cell therapy, the forms of the biomaterials that are usually applied to the damaged tissue are implantable scaffold and injectable gel.

1.3.1 *Natural materials*

Fibrin is an insoluble protein which is responsible for clotting blood after bleeding. The formation of fibrin is a result of polymerization of fibrinogen under clotting enzyme thrombin. For tissue regeneration, fibrin scaffold and fibrin glue are widely used [38, 39]. The 3D gel which is the reaction product of thrombin on fibrinogen can be achieved within 10 to 60 seconds depending on the concentrations of components and crosslinking agent. The content of fibrinogen and the stiffness of the scaffold can be controlled to match the application requirement. As a consequence, fibrin scaffold is suitable for cell culture and implantation into host tissue.

Alginate is an anionic polysaccharide copolymer of mannuronate and guluronate residues. Alginate is water absorbable, biocompatible and can turn to a hydrogel with the addition of divalent cations, such as Ca$^{2+}$. In addition, alginate is a FDA approved material which can be used for human wound healing, cell encapsulation, and transplantation [40]. Alginate gel were injected into infarct rat heart to prevent remodeling by Landa et al. [41].

Collagen is a biocompatible and biodegradable structural protein which widely exists in human and animal bodies, and can be isolated and purified from human and animal tissue. Collagen, with simple crosslinking, form a hydrogel in a relatively short time. The RGD containing domain in collagen can promote cell attachment and cellular interactions.
Therefore, collagen gel can be used as an injectable material to directly deliver cells or be applied for cell culture and later implanted material. By injecting collagen gel together with stem cells into infarct heart, myocardial function was preserved and vascularization was promoted [42, 43].

In addition to the above stated natural materials, there are other natural materials applied in heart and brain tissue engineering. Matrigel has perfect cell adhesive property which can be used as an attachment substrate for embryonic stem cell culture to proliferate and differentiate [44]. Chitosan is a cationic polysaccharide copolymer which can be made into chitosan hydrogel with porous structure and chitosan scaffold with nanofibrous structure. It is reported that chitosan gel can induce angiogenesis and heal the wound [45].

1.3.2 Synthetic materials

Different from natural biomaterials, synthetic materials are developed via chemical methods. Most synthetic materials are polymers which are synthesized via polymerization methods. Thus, the physical and chemical properties of the materials can be controlled in the preparation process. The capability of endowing synthetic materials with functional molecules (peptides, growth factors and drugs) as well as the tunable materials’ properties are advantages for using synthetic materials in comparison with naturally derived biomaterials, which are unlikely amenable for modification. Therefore, synthetic biomaterials are extensively studied and applied for tissue engineering.

Poly (α-hydroxy esters) is a category of polymers that are usually used for cell delivery. It includes poly (glycolic acid) (PGA), poly (lactic acid) (PLA), and poly (lactic-co glycolic
acid) (PLGA) which is a copolymer of PLA and PGA. PLA, PGA and PLGA are all FDA approved for tissue regeneration [46]. They are usually fabricated into biodegradable scaffolds with considerable mechanical properties. The mechanical properties and degradation rate are tunable by changing the ratio of PLA and PGA. Xiong et al. [47] studied the feasibility of transplanting NSCs seeded PLGA scaffold in neural repair. In the study, transfected NSCs were seeded into the scaffold and cultured for 14 days. High cell engraftment and synaptic structures were observed and differentiation into neurons were detected. PLGA scaffolds with bFGF were used to promote cardiac angiogenesis by Wang et al. [48]. Polycaprolactone (PCL) is another biocompatible materials used to fabricate a scaffold by electrospinning method. Singh et al. [49] used porous PCL scaffolds to deliver endothelial progenitor cells (EPCs) to promote vascularization. With presence of growth factor, the blood vessel formation was accelerated in vivo. Implantable PCL nanofibrous scaffolds were fabricated for neural tissue engineering by Gupta et al. [50]. In addition, biodegradable polyurethaneurea scaffolds developed by Guan et al. [51] were capable for cell seeding and therefore can be applied to tissue engineering.

Some categories of synthetic polymers can form hydrogels, which are networks of crosslinked structures. Hydrogels are hydrophilic materials which have relatively high water content. PEG hydrogel is used extensively for tissue engineering. PEG hydrogel is made by crosslinking polyethylene glycol methacrylate (PEG-MA)/dimethacrylate (PEG-DMA) under UV exposure with addition of photoinitiator (Irgacure 2959) [52]. PEG hydrogel was used to culture neural progenitor and endothelial cells to promote functional blood vessel formation in vivo [53]. Poly (2-hydroxyethyl methacrylate) (poly-HEMA)
hydrogel is another widely used hydrogel similar to PEG hydrogel. There is another special type of hydrogel which is thermosensitive. This kind of hydrogel is based on N-substituted polyacrylamides, such as poly(N-isopropylacrylamide) (PNIPAAm). Li et al. [54] synthesized a thermosensitive hydrogel which was a copolymer of N-isopropylacrylamide (NIPAAm), acrylic acid (AAc), dimethyl-gamma-butyrolactone acrylate (DBA), and 2-hydroxyethyl methacrylate-poly(trimethylene carbonate) (HEMPTMC) monomers. Live cell tracker (CMFDA) labeled MSCs were cultured in the hydrogel and cell proliferation was observed.

In addition, many other synthetic materials were developed for cardiac repair and brain repair such as poly (ethylene oxide)-b-poly(propylene oxide)-b-poly(ethylene oxide) (PEO-PPO-PEO) [55], poly(D-lysine) (PDL) [56], etc. Biomaterials were also prepared by conjugating or copolymerizing these materials such as MPEG-PCL-MPEG [57], PEG/PCL hydrogel fiber [58], etc.

1.4 Cell survival issues and solutions

1.4.1 Mechanisms and factors

Stem cell therapy for infarct heart and brain experiences dramatic cell death during the process of transplantation step and further recovery step. Due to the motion of heart, a large amount implant cells are squeezed out the implanted site, and the cells can also be lost in the circulation of both the heart and brain. Thus, the retention rate of transplanted stem cells is unsatisfactory.
The stem cells, when engrafted in the implanted site, stay in a harsh environment and large percent of the cells die in a short period of time afterward. The fundamental reason for cell death after implantation is the ischemic environment induced in the infarct heart and stroke brain. Under ischemic conditions, the oxygen level is extremely low and the nutrient supply from blood is limited. Both oxygen and nutrient delivery are essential factors to support cells to be alive. The harsh surrounding environment of ischemic conditions in the stem cell transplantation site results in massive cell death which is initiated by the ischemia pathway. Even worse the survival factors which promote cell survival are absent in the environment. Therefore, the efficiency of cell transplantation is extremely low and the efficacy of the stem cell therapy is limited.

Transplanted cell death can be a result of anoikis, apoptosis, and necrosis. In normal conditions, the cells adhere the surrounding tissue and there is specific interaction between cell and extracellular matrix (ECM). Only in a proper environment within a tissue can the transplanted stem cells grow and differentiate. Otherwise, apoptosis is initiated as a response and the cells are removed eventually. Anoikis is induced from apoptosis under conditions of weak or improper adhesion between cell and ECM or even cell detachment from the ECM. As a result, stem cells can hardly survive in an inappropriate ECM environment. In 1994, Frisch and Francis first reported cell death following anoikis mechanism because of lack of cell adhesion [59]. Epithelial cells cultured in adhesion-inhibited conditions leads to increased apoptosis. Therefore, anoikis can be considered an issue of designing of biomaterials.
Although apoptosis and necrosis are both considered to be the causes of significant cell death, necrosis can cause significant stem cell death within the first day after transplantation compared to apoptosis. Apoptosis is the process of programmed killing of the damaged cells of which the characteristic morphology change. This process happens during acute myocardial infarction and brain injury [60, 61]. For the stem cells transplanted to injured brain, apoptosis and necrosis induced caspase-mediated cell death was observed due to the damage that is a consequence of the following factors: oxidative stress, trophic factor withdrawal, or insertion of a needle. After NPCs were transplanted into traumatically injured brains, it was showed a caspase-mediated cell death peak at 3 days and a calpain-mediated cell death peak at 1 week [62].

Acute infarction in heart and brain lead to cell death and tissue damage. As a response, the dead cell are removed due to apoptosis. In the process, macrophages are introduced to the infarct region to remove the damaged tissue. At the same time, reactive oxygen species are released and free radicals levels are increased, which threaten the survival of the transplanted stem cells. This process is also accompanied with local infection and inflammatory response from the immune system. Large immune proteins induced from host immune system as a response to infection can present negative effects on the transplanted stem cells. Immunoglobulin G (IgG) is a four peptide chain protein (150kDa) complex antibody which is most commonly found in circulation. IgG has the ability of binding pathogens to protect the tissue from infection. Large amounts of IgG infiltration appeared in the ischemic infarct area in rat was found by Czurko et al. [63]. Even though inflammation is a self-healing mechanism of the body, inflammation can hinder cell
transplantation. The inflammation related factors can decrease cell proliferation and cause cell death. After MI and stroke, pro-inflammatory cytokines such as interleukins-1β and -6 (IL-1β and IL-6) and tumor necrosis factor-α (TNF-α) are secreted and the cytokines inhibit stem cell proliferation and differentiation [64, 65]. Thus, the cardiomyocytes and neural tissue regeneration are hindered.

1.4.2 Methods to improve cell survival

To increase the cell survival, the primary concern is to avoid cell loss during delivery. Saline based delivery medium is not an optimal choice because the cells have no attachment to ECM in saline and this can lead to anoikis-induced cell death. On the other hand, the viscosity of saline medium is low, like that of water. The stem cells delivered to infarcted heart have low retention rate due to the circulation and heart motion. Therefore, biomaterials are essential in the stem cell therapy to serve as delivery vehicle of stem cells. Typically, biomaterials are frequently used in the forms of scaffold or hydrogel, which are a biomimetically designed ECM which have the similar properties of natural ECM. Firstly, they provide the stem cells with a microenvironment similar to ECM for attachment. Secondly, biomaterials are designed to possess suitable physical and chemical properties to guide stem cells proliferation, differentiation, and integration with the host tissue [66].
1.4.3 Combine biomaterials and cell adhesion molecules

To avoid stem cell death caused by anoikis before and during transplantation, cell adhesion molecules should be introduced into the biomaterial cell delivery system to improve the cell attachment to non-ECM materials. Typically cell adhesion molecules are mixed with or conjugated to the biomaterial scaffold or gel and the enclosed suspended stem cells will have good cell-ECM interactions and cell viability. Karoubi et al. [67] studied the cell viability of MSCs in agarose with and without the addition of fibronectin and fibrinogen. The results showed an increase in cell viability after adding fibronectin and fibrinogen to the agarose capsule. In another study, fibrin glue is used to mix with cells and injected into infarcted heart. The retention of cells is significantly improved and survival of the cells is increased due to the contribution of fibrin, which served as ECM for the delivered cells [38]. Cook et al. [68] investigated the behaviors of cell adhesion and differentiation on several cell adhesion molecules (collagen I, collagen IV, fibronectin and laminin) and proved the increase of cell attachment on the modified surfaces. In addition, peptides, YIGSR/IKVAV and RGD, derived from cell adhesion domain of laminin and fibronectin can also be used to modify the biomaterials scaffold or hydrogel to form a biomimetic cell-interactive surface which can provide cell with affinity like ECM proteins exhibit. Jongpaiboonkit et al. [69] reported an increase (from 46% to 81%) of viability of MSCs which were cultured in a PEG gel with IKVAV/RGDSP modification compared with MSCs cultured in unmodified gel. Cell survival rate was observed to increase significantly when IKVAV or RGDA based cell adhesion peptides were used to modify other biomaterials, like poly(2-hydroxyethyl methacrylate) (PHEMA) hydrogel [70].
1.4.4 Combine preconditioning method with stem cells

Post-transplanted stem cells for stem cell therapy died due to major factors of ischemia and inflammatory cytokines in the infarct region. Preconditioning methods which pre-treated stem cells with ischemia or cytokines for cytoprotection is an alternative strategy applied to stem cells to alleviate apoptotic cell death by enhancing the cell tolerance to the effects of harsh microenvironment.

Murry et al. [71] first investigated the preconditioning method in 1986. This work showed that the ischemic preconditioning method, cyclic exposure to brief ischemia, and reperfusion are effective and potent cytoprotective measures in ischemic myocardium. Later in 1998, Maulik et al. [72] proved that ischemic preconditioning and reperfusion to ischemic heart can enhance survival due to ischemia adaption. Reduced oxygen consumption was found by Grund et al. [73]. Similarly, recent study from Ma et al. [74] indicated that ischemic preconditioning was a neuroprotective method for ischemic-induced brain injury. Furthermore, ischemic preconditioning was proved to enhance growth factor expression and cardioprotection [75, 76]. However, there was a contentious of protective mechanism and different proteins and pathways were proposed [77, 78].

Withdrawal of trophic factors in the infarct tissue results in cell apoptosis. And it is showed that growth factors can improve cell survival, differentiation, and proliferation in cardiac and neural repair [79]. Cytokine preconditioning activates the downstream signal transduction to enhance cell survival by treating cells with growth factors. Studies showed that cytokine preconditioning of cells with growth factor proteins significantly enhanced
cell survival [80, 81]. Preconditioned MSCs with SDF-1α can release anti-apoptotic and angiogenic cytokines. The endothelial progenitor cells, cardiomyocytes, and cardiomyoblasts survival, proliferation, and angiogenesis in ischemic heart were improved by VEGF and bFGF preconditioning which resulted in paracrine factor expression, minimized infarct size, and left ventricle remodeling. IGF-1 preconditioning of stem cells showed both cytoprotection and integration to host tissue in vitro and in vivo [82, 83]. In the studies, two major pathways, PI3K/Akt and MAPK/Erk1/2, which prevent cell apoptosis, were activated because of the interaction between IGF-I and IGF-1 receptor on the cell surface. Moreover, an in vitro study showed upregulated expression of connexin 43, which is responsible for enhancing cell engraftment. For further confirmation, improved cell survival and engraftment were observed after cells were transplanted into the infarcted rat heart.

1.4.5 Combine biomaterials and oxygen release systems

Oxygen is critical for cellular physiology, metabolism, signaling pathways, and cell engraftment. Hemoglobin and myoglobin are known to transport oxygen to tissues with a behavior of binding and releasing oxygen. The extremely low oxygen concentration in the infarction induced ischemic heart and brain results in significant transplanted stem cell death. Combining an oxygen release system with a transplantation system to augment the oxygen content in the target region is considered to be a feasible strategy to improve cell survival [84].
Inorganic peroxide based oxygen generation system can produce oxygen continuously. S.H. et al. [85] combined calcium peroxide based oxygen release particles with PLGA scaffold. The modified scaffold can sustain the release of oxygen for 10 days to enhance cell viability under hypoxic conditions. Because the myocardial functions and nervous functions are related with sodium and calcium ions. It is considered a potential risk that the diffusion of sodium and calcium irons from inorganic peroxide based oxygen releasing system may disturb the normal heart and brain functions. To avoid the ion effect, organic molecules pyridine endoperoxide oxygen release system was fabricated by Benz et al. [86] to enhance cell survival for up to 13 hours. Mallepally et al. [87] enclosed hydrogen peroxide into poly (methyl methacrylate) microcapsules to control oxygen delivery. Wang et al. [88] developed a porphyrin based hemoprotein (rHSA(FeP-Glu)) which can bind and release oxygen reversibly. However, the above methods can provide a controlled release oxygen within a day. Oxygen is required for both short and long term periods to enhance cell survival and angiogenesis. Abdi et al. [89] encapsulated hydrogen peroxide into PLGA microspheres to release oxygen for 7 days. Li et al. [90] proposed a hydrogen peroxide and PVP complex encapsulated PLGA microsphere as a long term oxygen release system which was proved to continuously generate oxygen for 2 week at a relatively high oxygen level. Cell viability was significantly improved in an oxygen release system under hypoxic environment in vitro culture. These works indicated the potential of oxygen release biomaterials for enhancing cell survival under hypoxic conditions and promoting neovascularization to restore blood flow.
1.4.6 Combine biomaterials and growth factors

Due to the disturbance of blood flow in the infarcted area, the transplanted stem cells and host tissue can hardly survive in the harsh environment with extremely low oxygen and nutrients. Seeking a method to stimulate neovascularization in the infarcted area together with stem cell implantation is a long term concern for tissue regeneration of heart and brain. Combination of growth factors and biomaterials is a viable strategy for cell delivery.

Whether growth factors can enhance cell survival and promote cell differentiation in acute MI heart and stroke induced ischemic injured brain has been well studied and proven. To solve the critical problems of ischemic effect in the infarcted area and increase the efficiency of stem cell therapy, the chosen growth factors should have the ability to help transplanted stem cells to avoid apoptosis and promote angiogenesis. As a result, the issue of ischemia induced cell death can be addressed in both short and long term considerations. Moreover, specific growth factors can promote stem cell differentiation to replace the dead functional cells, such as cardiomyocytes and neurons, and the function of heart and brain can be recovered to some extent. Among the large family of growth factors, fibroblast growth factor (FGF) [91], platelet derived growth factor (PDGF) [92], and vascular endothelial growth factor (VEGF) [93] etc. are proven to promote cell survival, proliferation, and stimulate angiogenesis. Growth factors can bind to specific receptors on the target cell surface and serve as signaling molecules to activate cellular pathways, which improve cell survival and differentiation.
1.4.7 Combine biomaterials and anti-inflammation molecules

Immune system rejection lowers the transplanted cell survival rate. Large protein antibody like IgG and pro-inflammatory cytokines like TNF-α and IL-1 are responsible for the massive cell death [94]. Even though biomaterials are usually used as carriers for cell delivery, inflammatory cytokines, immune cells, and antibodies can infiltrate into the matrix and contact with cells to trigger the apoptotic pathways. Combination strategies of specially designed biomaterial barrier and anti-inflammatory molecules should provide immunoprotection for transplanted cells.

From the pure biomaterial aspect, semi-permeable biomaterials [95] can be synthesized and prepared. By controlling the pore sized, the biomaterial matrix forms an immune-isolation barrier to prevent the infiltration of the large mediators, like IgG, from the host immune system [96]. As an immunoprotection method, the stem cell encapsulated materials, which block the penetration of large immune molecules, were used therapeutically to transplant stem cells into host tissue, like nervous tissue [97]. Many synthetic and natural materials were investigated for this approach. In many studies [98-100], semi-permeable PEG hydrogels were used to encapsulate cells and effectively isolated transplanted stem cells from the immune cells and antibodies attack. Cell survival was promoted as a result in vitro and in vivo.

However, small cytotoxic molecules secreted by neutrophils and macrophages, such as reactive oxygen species (ROS), TNF-α and IL-1β, can pass through the biomaterial barrier, diffuse in the matrix, and lead to cell death and additional cytokine secretion [101, 102]. By increasing the degree of crosslinking and the concentration of materials, the infiltration
of the small molecules is suppressed. Concerns of reduced water content and impeded nutrients which threaten cell survival imply these to be an unlikely approach. Modification of biomaterials with functional molecules was another way to address this tissue. Hume et al. [103] incorporated superoxide dismutase mimetic (SODm) into size exclusion PEG hydrogel to form a functional gel and the encapsulated cells showed around 60% of metabolic activity while unmodified biomaterial group showed none. Therefore, the transplanted cells in the materials were protected from oxidative damage from ROS. Lin et al. [104] provided cells with TNF-α cytokine protection by encapsulating cells in PEGDA hydrogel conjugated peptide WP9QY (YCWSQYLCY). The peptide functionalized hydrogel showed high TNF-α binding ability. Further studies by Su et al. [105] modified armed PEG hydrogel with anti-inflammatory peptides and RGD peptide for early protection of encapsulated cells in the presence of TNF-α, IL-1β, and INF-γ. In both studies, the encouraging results showed that cell survival and engraftment improved and cell function was prolonged.

1.4.8 Genetic modification of stem cells

As discussed previously, growth factors are proven to show the effects of not only enhancing stem cell survival, proliferation, and differentiation, but also promoting angiogenesis and neurogenesis. However, there exists a biological concern about the stability of growth factors. Most of the growth factors which can activate cell survival signaling pathways are proteins which have a relatively short bioactive half-life. It is well known that growth factors can upregulate the paracrine factor expression of transplanted
stem cells which in turn can enhance cell engraftment and proliferation. Thus, the unstable effect can be addressed by genetic modulation of stem cells for growth factor expression and pro-survival molecule expression.

Studies [106, 107] on transferring encoded genes of growth factors and anti-apoptotic factors to stem cells before transplantation in order to prevent cell death due to apoptosis. In these studies, cells which can express angiogenic growth factors were applied for MI heart treatment. The genetically modulated cells were able to secret autocrine and paracrine growth factors which were consistent with the transgenes. As a result, cell survival and angiogenesis were improved, and some heart function was recovered as left ventricular remodeling was limited. Matsumoto et al. [108] transfected VEGF gene to MSCs and injected the modified MSCs into MI rat heart. High expression of VEGF was detected and cell death was reduced because of the activation of survival signaling after 1 week. Infarct size was improved and capillary density was significantly increased after 28 days. IGF-1 gene modulated and dual growth factor genes modulated stem cells in stem cell therapy also showed improved cell survival [109-111]. For stroke treatment, Lee et al. [112, 113] showed genetically modified NSCs, which overexpress brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and VEGF enhanced cell survival, promoted angiogenesis and improved function of damaged brain in mouse.

1.5 Discussion

To treat myocardial infarction heart disease and ischemic-induced brain injury, many approaches have been proposed and put into clinical application. Among them, stem cell
therapy is considered a potent and promising therapeutic method. However, the efficacy is limited as an extremely low cell survival rate was observed in the clinical trials. A major reason which lead to cell death in both cases is the induced ischemia. Oxygen deprivation and inflammatory response result in apoptosis of host cells and implanted cells. Moreover, transplanted cells cannot survive without an adequate nutrient supply.

The choice of proper stem cell types for specific delivery systems to fit these requirements is crucial to ensure cell survival and differentiation in the harsh ischemic environment. Even though ESCs are considered an ideal stem cells type for the stem cell therapy, there are some studies shows that undifferentiated ESCs can form a tumor after transplantation. Alternative stem cells are therefore favored. It was doubted that neurons that differentiated from MSCs had no normal functions. Further studies are required to clarify the possibility of nervous regeneration by these stem cells.

There is evidence that showed that a large percent of the transplanted cells were lost in a short time after implantation. Widely studied injectable gel delivery systems including collagen gel and fibrin gel fit these criteria because of their low viscosity and long gelation time. NIPAAm-based synthetic thermosensitive and biodegradable hydrogels have a fast gelation rate (in seconds) compared with that of other injectable hydrogels. Injected cell retention rate was significantly improved in comparison with saline and collagen gel injection systems. Some gel formations require UV radiation, pH changing or ions addition. The effect of factors on cells need to be clarified. In addition, biomaterial type, degradation products toxicity, dose, and timing must be well studied before the clinical application.
According to the approaches which enhanced transplanted cell survival, hydrogen peroxide based oxygen release system proved to maintain oxygen release for a long time. This method involved hydrogen peroxide diffusion into the environment before catalysis by enzyme to oxygen and water. There is possibility that hydrogen peroxide contact with cells and host tissues may induce an inflammatory response. Further studies on toxicity of this effect, method optimization, and in vivo study are need to support the feasibility of clinical trials. Ischemic preconditioning may damage cells in the process and the implanted cells may not survive under ischemic conditions for a long enough time which is needed for cell differentiation and angiogenic establishment. The operation of genetic modulation process on cells is complicated and may raise safety concerns.

From a clinical view, safety and efficacy are still paramount issues. Additional studies on animals are required in order to develop a reliable cell-biomaterial delivery system with a long-term investigation. Additionally, it is necessary to elucidate the mechanism responsible for these results.
CHAPTER 2: An injectable hydrogel based basic fibroblast growth factor (bFGF) release system to augment cardiosphere-derived cells (CDCs) survival and angiogenesis under ischemic conditions

2.1 Introduction

Myocardial infarction (MI) is a leading cause of death worldwide [114]. Myocardial infarction (MI), commonly known as heart attack, leads to irreversible death of heart muscle due to the lack of oxygen and nutrient supply [115]. In the heart, cardiac muscle contraction pumps nutrient- and oxygen-rich blood to supply the entire body while coronary arteries (CA) supply blood for the cardiac muscle [116]. When blood supply was interrupted due to CA narrowing or clotting, heart cells die within minutes, leading to myocardial infarction (MI). After MI, the death of muscle cells triggers a remodeling cascade. The infarcted area is gradually filled with collagen-containing scar tissue to withstand the higher pressure during the contraction cycle (systole). As the scar tissue becomes thinner, the heart function further decreases; this finally manifests as congestive heart failure (CHF) [117].

Current clinical intervention for MI was mainly concentrated on coronary reperfusion, which aimed to reintroduce oxygen in the infarcted heart to lower cell death. Reperfusion therapy, however, was a conservative method that did not involve new cardiac muscle
regeneration because adult cardiomyocytes were non-regenerative. Delivering stem cells into the infarct heart tissue for cardiac regeneration is accepted to be a promising therapeutic approach [118]. However, when a myocardial infarction occurs, blood flow is interrupted and ischemic environment is presented in the affected cardiac tissue. As a result of shortage of oxygen and nutrition supply, large amounts of cardiomyocytes die. Cardiomyocytes cannot regenerate themselves [119, 120]. Stem cell therapy is considered to be a potential way to treat MI because stem cells have the ability of self-renewal, proliferation and differentiation into many cell types [39]. To fully regenerate a functional heart tissue, cardiomyocytes, smooth muscle cells and endothelial cells etc. are required. To study the possibility of implantation and the ability of differentiation, many types of stem cells were investigated, including induced pluripotent stem cells (iPSCs) [121], mesenchymal stem cells (MSCs) [122], intrinsic cardiac stem cells (CSCs) [123], embryonic stem cells (ESCs) [124] and cardiosphere-derived cells (CDCs) [125]. Among these cells types, CDCs are regarded to be a promising cell type for stem cell therapy. CDCs can be easily isolated from the explant of exocardium biopsies and preserved a high proliferation rate [126]. A considerable number of CDCs can be acquired in relatively short culture period. CDCs were proved to be able to differentiate into cardiomyocytes in vitro and in vivo. In addition, CDCs have a higher differentiation capability than MSCs [127]. There is no ethical concerns and potential danger like ESCs, which also differentiate into other non-needed cell types for cardiac regeneration.

By introducing stem cells into damaged tissue area, the cells integrate with the native heart. The engrafted stem cells could proliferate and differentiate into target cells to
regenerate the lost tissue so as to restore the heart functions. Many clinical trials are ongoing to deliver stem cells into the heart. A commonly used cell delivery approach in the therapy is to suspend cells in a buffer and then inject the mixture into the heart infarct area. While the regeneration behavior is observed on the engrafted cells, the regeneration efficacy remains low and the injected cells have failed to show a satisfied engraftment rate [128, 129].

The efficacy of the therapy is considered to be a critical issue when stem cell therapy is applied for cardiac regeneration. Due to the motion of beating heart, few stem cells (less than 10%) can engraft in the injected tissue area while large quantities of injected stem cells are squeezed out and lost in circulation [2, 39]. Because of inadequate biocompatibility of encapsulating materials and ischemic environment, a large percent of engrafted cells dies a few days after injection. Another possible reason is the harsh environment at the infarcted area – low oxygen and low nutrient which are caused by the blockage of the coronary artery. Massive cell death is initiated by the ischemic pathway. All of these factors greatly limit the survival of the delivered cells. Only a small percent of surviving stem cells is observed to differentiate. [3, 130]

The cell death could be a result of anoikis, apoptosis, and necrosis. In normal conditions, the cells adhere to extracellular matrix (ECM) which is a proper environment for cell growth and differentiation. Anoikis is induced from apoptosis under conditions of weak or improper adhesion between cell and ECM or even cell detachment from the ECM. In 1994, Frisch and Francis first reported cell death following anoikis mechanism because of lack of cell adhesion [59]. Necrosis can cause significant stem cell death within the first
day after transplantation. Apoptosis is the process of programmed killing of the damaged cells during acute MI [60, 61].

Previous studies in our group reported that it was feasible to use injectable thermosensitive hydrogels as cell carriers for tissue regeneration [130-132]. There are several advantages of using injectable thermosensitive hydrogel. It has good mobility and is easily to operate which means it can be surgically injected with minimal invasion and filling the irregularly shaped defects. Meanwhile, it quickly solidifies in an in vivo environment, forms tissue constructs and preserves encapsulated cells in the injection site as well as providing a ideal biological microenvironment for cells growth. Therefore tissue regeneration are able to occur locally [133].

In this work, we hypothesized that the sustained released of growth factor basic fibroblast growth factor (bFGF) could promote survival rate of encapsulated CDCs under ischemic condition and further promote cardiac regeneration and blood perfusion recovery. bFGF was proven to stimulate the paracrine effect and trigger the cell survival pathway which enhances cell survival and proliferation [134]. Many studies have reported that bFGF is able to enhance stem cell survival, differentiation and promote angiogenesis [131, 135, 136]. However, encapsulation efficiency of bFGF and bioactivity of the long time released bFGF are limited. The properties and the standard processing conditions of hydrogel provides good bFGF encapsulation efficiently. Our previous study of bFGF indicated that bFGF stabilized by heparin was able to maintain bioactivity for a relatively long period of time [131].
In order to improve stem cell survival and allow cardiac tissue regeneration for short term and long term periods, by loading bFGF into hydrogel a thermosensitive hydrogel based bFGF releasing system was developed as our objective. Based on the previous study, viscous, NIPAAm based injectable hydrogels can be used as cell carriers to efficiently hold cells in tissue, avoiding cell loss due to low viscosity medium [137]. At the same time, the hydrogel was expected to keep bioactivity of the encapsulated bFGF and release bFGF for a relevantly long period of time as well. The released bFGF was able to augment the transplanted cell survival rate, stimulate paracrine effect and promote angiogenesis for long term cell survival and blood vessels development for newly regenerate tissues.

2.2 Materials and methods

2.2.1 Materials

2-hydroxyethyl methacrylate (HEMA, Alfa Aesar) was purified by passing through a column loaded with inhibitor remover. N-isopropylacrylamide (NIPAAm, TCI) was recrystallized with hexane three times before use. Benzoyl peroxide (BPO), sodium methoxide (NaOCH₃), methacryloyl chloride, lactide (Sigma), bFGF (Peprotech), and Heparin (VWR) were used as received.

2.2.2 Hydrogel polymer synthesis

Biodegradable monomer acrylate polylactide (APLA) was synthesized by a two-step method [138]. Polylactide (HO-PLA-OCH₃) was synthesized by NaOCH₃-initiated ring-
opening polymerization of lactide. Lactide (3,6-Dimethyl-1,4-dioxane-2,5-dione, 100g, 0.694mol) was dissolved in 150 mL of dichloromethane, to which a NaOCH₃ solution containing 2 g NaOCH₃ (0.037mol) in 20 mL of methanol (0.494mol) was added under vigorous stirring. The reaction proceeded for 2 h at 0°C (on ice) before the solution was sequentially rinsed with 0.1M HCl and deionized (DI) water. The organic phase was isolated and dried over anhydrous MgSO₄. The solvent dichloromethane was removed by rotary evaporation at 60°C to obtain around 90 g HO-PLA-OCH₃. APLA was synthesized by adding 38mL acryloyl chloride (0.4mol) dropwise into the HO-PLA-OCH₃ (90 g, 0.39mol) solution in 150 mL of dichloromethane containing 65 mL (0.47mol) of trimethylamine at 0°C in a 1h period of time. After the react was proceed at room temperature overnight, the solution was filtered to remove precipitate and was then rinsed sequentially with 0.2 M Na₂CO₃, 0.1M HCl, and saturated NaCl solution. The bottom organic phase was isolated and dried over anhydrous MgSO₄. The solvent dichloromethane was removed by rotary evaporation at 40°C to get the raw product of APLA, which was finally purified by flash chromatography. The raw product was flashed through a silica gel column by a mixed solvent - Ethyl acetate/Hexane (2/1). The solvent was removed by rotary evaporation. The final yield was about 60%. The structure of APLA and number of repeating unit were confirmed by ¹H-NMR.

The hydrogel was then synthesized by using free radical polymerization method. NIPAAm, APLA, and HEMA were copolymerized at a molar feeding ratio of 86/4/10 (Figure 2.1). In brief, a 250mL, three-neck round bottom flask was added with NIPAAm (4.96g), APLA (0.544g), and HEMA (0.664g) monomers at decided ratio (86/4/10).
120mL solvent dioxane was poured into the flask. After the monomers were fully dissolved, initiator benzoyl peroxide (BPO, 24.7mg) was added and the polymerization reaction was proceeded in 70°C oil bath overnight with nitrogen protection and magnetic stirring. The polymer was precipitated out in cold hexane. Then the polymer was further purified twice by dissolving in tetrahydrofuran (THF) and precipitating in dry ethyl ether. The polymer was allowed vacuum dried for 24 hours before use. The structure of the hydrogel polymer and ratio of components were confirmed by $^1$H-NMR.

2.2.3 Hydrogel properties characterization

Hydrogel solutions were prepared by dissolving the hydrogel polymer in Dulbecco’s modified phosphate buffer saline (DPBS, pH = 7.4) at a concentration of 20%(w/v) at 4°C (The concentration of hydrogel solution is 20% in the following content if no specific comment is made.). The thermal transition temperature of the hydrogel solution and degradation product were measured by differential scanning calorimetry (DSC). The temperature range was set from 0 to 60°C and the heating rate was controlled at 10°C /min. A 26-gauge needle, which is commonly used in animal surgery, was used to test the injectability of the hydrogel solution at 4°C. The gelation time of the hydrogel solution at 37°C was measured by an Olympus IX71 microscope equipped with a temperature controllable chamber. The hydrogel solution was dropped onto a pre-warmed glass slide on the microscope stage in the chamber. A video of the gelation process was recorded. The time was determined based on the video when the hydrogel solution was transmitted to opaque.
The solid hydrogel was acquired after the hydrogel solution was gelled in a 37°C water bath until equilibrium water content was reached within 2 hours. The hydrogel was then applied for water content measurement and tensile test. After the solid gel (in microcentrifuge tube) reached equilibrium water content, the supernatant was removed from the tube. The wet weight of the hydrogel was measured by subtracting the weight of tube+wet gel and the empty tube and recorded as \( w_1 \). Then the wet gel with the tube was freeze dried. The lyophilized hydrogel weight was measured by subtracting the weight of tube+dry gel and the empty tube and recorded as \( w_2 \). The water content was calculated as:

\[
\text{Water content (\%) = } \frac{w_1 - w_2}{w_2} \times 100\%.
\]

The mechanical properties of the hydrogel were tested with an Instron tensile tester with a 50lbs load cell and a cross-head speed of 50 mm/min. Strip shaped samples were cut from the solid hydrogel. Width and thickness of each sample was measured. The hydrogel modulus was calculated based on the data collected from the elastic deformation region of the stress-strain curves and the dimensions of the sample. At least 5 gel samples were tested.

To determine hydrogel degradation, around 0.04g solid hydrogels were placed in 2mL microcentrifuge tubes with 200μL DPBS in a 37°C water bath for 8 weeks. The remained weight of hydrogel at each time point was measured as \( w_3 \) after they were freeze-dried. The weight of sample before degradation (week 0) was \( w_4 \). The weight remaining was calculated as: Weight remaining (\%) = \( \frac{w_3}{w_4} \times 100\% \).

The degradation product toxicity was test with MTT assay with degradation product NIPAAm-AAc-HEMA which was synthesized. Cardiac fibroblast cells were used and seeded in 96-well plate at a cell density of \( 2 \times 10^5 \) cells/well with 200μL fibroblast
culture media in each well. The plate was incubated in 5% CO₂ and 37°C incubator for 24 hours and then the culture medium was replaced. The degradation product solution (in DPBS) was added to each well to final concentration of 1mg/mL, and 5mg/mL. The plate was then incubated in the same incubator for additional 48 hours. 20μL MTT solution (5mg/mL) in DPBS was prepared and added to each well. After the plated was incubated for 4 hours, the medium was removed carefully and 200μL DMSO was added to each well. The plate was shaken carefully until the precipitate in each well was fully dissolved. The plate was read at 560nm and background was subtracted at 670nm.

2.2.4 bFGF releasing hydrogel fabrication

Hydrogel solution was prepared following the described method. To make a bFGF releasing hydrogel system, heparin was first mixed with the hydrogel solution at a concentration of 1mg/mL. Then bFGF was added to the hydrogel solution to acquire a final bFGF concentration of 50μg/mL. The mixture was stirred at 4°C for several hours to reach a homogenous solution. 0.2mL hydrogel solution was transferred into the 2mL microcentrifuge tube which was incubated in 37°C water bath and allowed gelation for 30min.

2.2.5 bFGF release kinetics and bioactivity of the released bFGF measurement

To measure the amount of released bFGF, the 0.2mL bFGF releasing hydrogel solution was placed in each 2mL microcentrifuge tube. After incubated in 37°C water bath for
30 min, the supernatant in the tube was replaced with 0.2 mL DPBS as release buffer, and the supernatant was saved in a microcentrifuge tube and stored in -20°C refrigerator. The bFGF release was conducted at 37°C water bath for 28 days. At time point 8h, Day 1, 3, 5, 7, 14, 21, 28, the release buffer was collected in a microcentrifuge tube and stored in -20°C refrigerator and 0.2 mL new fresh release buffer was added. The bFGF concentration in the release buffer was determined by a bFGF ELISA kit (Peprotech). Release medium from the hydrogel without loading bFGF was used as control.

Bioactivity of the released bFGF was evaluated by rat fibroblast proliferation. The cells were cultured with Dulbecco’s Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) in incubator under normal culture conditions. Rat fibroblast cells were seeded to a 96-well plate at a density of $2 \times 10^5$ cells/well and 0.2 mL culture medium was added to each well. After the plated was incubated for 24 h in the incubator, the culture medium was replaced with the collected released bFGF medium supplemented with 5% FBS. After 48 h of incubation, cell viability was measured by MTT assay. 5 mg/mL MTT solution in DPBS was prepared and 20 μL MTT solution was added to each well. After 4h incubation at 21% O2, 5% CO2, and 37°C, the medium was carefully removed and 200 μL DMSO was added to each well. The plate was shaken carefully until the precipitate was fully dissolved. The plate was then read at 560 nm and subtract background at 670 nm. Release medium from the hydrogel without bFGF loading and 1 ng/mL bFGF loading were used as controls. Relative cell viability was normalized to the MTT absorbance of the release media from the 1 ng/mL bFGF loading. In addition, pure hydrogel and bFGF loading hydrogel were injected subcutaneously in mice. After 2 weeks, the mouse tissues
with hydrogels were fixed by 4% paraformaldehyde at 37°C for 1h and then rinsed with warm DPBS. Each tissue sample was embedded with OCT in a plastic mold and the mold was placed in -80°C freezer to allow OCT frozen. The sample was frozen sectioned at 10 microns afterwards. To visualize the blood vessels, immunostaining method was used. The sections were firstly stained with primary antibody CD31 (Abcam) overnight followed by fluorescent secondary antibody for 1h. The nucleus was stained with Hoeschst 3342. Then the staining slides were watched under FV1000 filter confocal microscope and images were taken for further analysis. The CD31 positive tubular structure in the images was regarded as blood vessel. The blood vessels were compared to evaluate the bioactivity of the released bFGF.

2.2.6 Cardiosphere-derived cells culture

Mouse CDCs were isolated from the atrium of the C57BL/6 mouse following previously established method [125]. The medium used for CDCs culture contained Iscove’s Modified Dulbecco’s Medium (IMDM, Invitrogen), 10% fetal bovine serum (FBS, Atlanta Biologicals), and 1% Penicillin-streptomycin (Invitrogen). The cells were cultured in T-175 cell culture flasks in the incubator at 21% O₂, 5%CO₂, and 37°C. Fresh culture medium was replaced every three days and CDCs were passaged when 90% confluence was reached. To encapsulate cells into hydrogel, we used CDCs at passage 13 - 15 which were proven to preserve stem cell phenotype and multipotency.
2.2.7 Encapsulation of CDCs into bFGF releasing hydrogel

The bFGF releasing hydrogel solution was prepared by stirring hydrogel polymer in DPBS at a concentration of 20wt% under 4°C overnight. Heparin (1mg/mL) and bFGF (50μg/mL) were added after the hydrogel solution was sterilized under UV light for 30 minutes on ice. The digested CDCs were suspended in small amount of DPBS and added into the hydrogel solution to reach a final cell density of 8 million/mL. To thoroughly mix the cells and hydrogel solution, the mixture solution was pipetted at least 20 times with a pre-cooled 1mL syringe.

2.2.8 CDCs survival under ischemic conditions in bFGF releasing hydrogel and generation of paracrine effect

CDCs were first labeled with live cell tracker CM-DiL (Invitrogen) following the provided protocol. Then the labeled CDCs were encapsulated in bFGF loaded hydrogel solution. 0.2mL CDCs, bFGF and hydrogel solution mixture was transferred into each 1.5mL microcentrifuge tube and the gel mixture was allowed gelation in 37°C water bath for 30 minutes. After the gelation, the supernatant was replaced with 0.2mL culture medium without FBS and a hole was punched on top of the tube. The tubes were incubated in hypoxia incubator (1% O₂, 5%CO₂, 37°C) for 7 days and the culture medium was replaced every three days. After 1, 3 and 7 days of culture, the gels were digested by 10mg/mL papain solution at 37°C for 24 hours. Cell survival was quantified by dsDNA content (for live cells) which was measured by a Quant-iT™ PicoGreen dsDNA Assay Kit (LifeTechnology), following the manufacture provided protocol.
Gene expressions for paracrine factors were characterized by real-time RT-PCR. RNA was isolated from the gels using TRIzol (Sigma) following the protocol provided by Sigma. The quality of RNA was measured by NanoDrop and then used for cDNA synthesis with a High Capacity cDNA Reverse Transcription kit (ABI). Primers of forward and reverse pairs of the platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and insulin-like growth factor 1 (IGF1) were selected for gene expression study. The sequences, melting temperature, and expected product sizes are listed in Table 2.1. Real-time RT-PCR was performed three times for each sample with Maxima SYBR Green/Fluorescein master mix on an Applied Biosystems 7900 system. β-actin was used as the housekeeping gene. Fold increase was calculated using a standard ΔΔCt method.

2.2.9 Injection of CDCs loaded bFGF releasing hydrogel into rat heart after acute MI

For in vivo study, acute MI model was made by ligating left coronary artery of rat heart. After 30 minutes, the system was injected through a 26-gauge needle into infarct area. CDCs were labeled with live cell tracker CM-DiL before injection. A total of 0.2mL mixture hydrogel solution with CDCs concentration of 8 million/mL was injected into the infarct area at 5 different injection sites with 40μL for each injection. The rat hearts were harvested after 2 weeks of implantation. The hearts were washed with DPBS three times and fixed with 4% paraformaldehyde at 37°C for 2 hours. The fixed hearts were sectioned at the infarct area and sent for paraffin embedding and sectioning at 5 μm.
For immunohistochemistry, the sections were blocked by 10% goat serum with 0.3% Triton X-100 at 37°C for 1h. Then the sections were first stained with anti von Willebrand factor (vWF) (abcam), anti Ki67 (abcam) and anti-myosin heavy chain (MHC) (abcam) primary antibody under 37°C overnight and then with corresponding fluorescent secondary antibody staining for 1h at room temperature. The slices were imaged using an Olympus FV1000 confocal microscope. Angiogenesis and cell differentiation (MHC positive cells) in the infarction tissue area was evaluated from the images. Blood vessels were recognized as tubular structures positively stained with vWF. Live cell, Ki67 and MHC positive cell numbers were calculated based on at least five different images. The left ventricle wall thickness of the infarct heart was calculated based on at least 5 sites on images of hematoxylin and erisin (H&E) stained slides.

2.2.10 Statistical methods

Data are expressed as mean ± standard deviation. Statistical comparisons were performed by ANOVA using JMP. Significant difference is defined as p < 0.05.

2.3 Results

2.3.1 Hydrogel polymer synthesis and properties characterizations

The hydrogel polymer was synthesized by copolymerization of NIPAAm, APLA and HEMA using free radical polymerization method at a feed ratio of 86/4/10. The synthesis process was showed in Figure 2.1. The structures of the synthesized monomer APLA and
hydrogel polymer were confirmed by $^1$H-NMR spectrum (Figure 2.2 and Figure 2.3). From the spectrum, the molecular ratio of each component in the polymer NIPAAm/APLA/HEMA was determined as 86/4.2/9.8 which was consistent with the feed ratio.

The 20wt% hydrogel solution was in liquid form and flowable at 4°C. It was able to inject through a 26-gauge needle which is usually used in surgery of tissue for cells injection (Figure 2.4). The hydrogel solution possessed a thermal transition temperature of 26.5 ± 0.2°C. The hydrogel was able to solidify under 37°C in 7 second. The short gelation time help ensure the gel retain in the heart after injection. After equilibrium the gel solution in 37°C, the soft gel had a water content of 48.7 ± 4.17 %. It showed high level of flexibility with a Young’s modulus of 35 ± 5 kPa. When the gel was incubated at 37°C in DPBS, it degraded slowly and had a weight remaining of 93.3% after 8 weeks (Figure 2.5). The degradation product was soluble at 37°C because the thermal transition temperature of degraded hydrogel raised to 42°C, higher than 37°C. The degradation product was proved to be non-toxic to cells (Figure 2.6).

2.3.2 Release kinetics of bFGF

The bFGF was loaded into the developed hydrogel at a concentration of 50μg/mL. The encapsulated bFGF (50μg/mL) was able to release from the hydrogel sustainably for a time period of 28 days. The cumulative bFGF release curve was showed in Figure 2.7. A burst of bFGF release was observed in the first 4 days and then the release rate slowed down but
continuously released until Day 28. Interestingly, a linear releasing behavior was observed after Day 7.

2.3.3 Bioactivity of released bFGF

The bioactivity of released bFGF was evaluated by measuring the cardiac fibroblasts growth. Release media from hydrogel without bFGF and 1ng/mL bFGF were used as controls. 1ng/mL bFGF was used as control because bFGF at this concentration was reported to stimulate fibroblasts growth significantly. In Figure 2.8, the released bFGF at every time point preserved bioactivity. Cardiac fibroblasts which were cultured in medium containing the released bFGF had higher relative cell viability than those controls. This indicated that the released bFGF was able to promote the growth of fibroblasts. Interestingly, the bioactivity of released bFGF was not lowered with increasing the releasing longitude. Furthermore, the bioactivity and function of released bFGF were studied in vivo. Hydrogels with and without bFGF encapsulation were injected subcutaneously in mouse and the tissue attached to the hydrogel were collected for immunohistochemistry analysis. In Figure 2.9, blood vessels were indicated by CD31 staining. By calculating vessel numbers from the images, increased blood vessel formation was observed with bFGF loaded hydrogel injection than the one with the pure hydrogel injection as showed in Figure 2.10. The results of angiogenesis observation demonstrated that the bFGF released from the hydrogel remained bioactive.
2.3.4 CDC survival in hydrogels with bFGF release under ischemic condition in vitro

Effect of bFGF releasing hydrogel system was evaluated by culturing CDCs in the bFGF loaded hydrogel in vitro. CDCs were mixed with hydrogel solution with addition of bFGF/haprin, cultured in a hypoxic incubator (1% O₂, 5%CO₂, 37°C) and supplied with no FBS culture medium which mimicked the ischemic environment in the infarct heart. The gelation time at 37°C remained to be around 7 seconds.

The dsDNA content was measured to quantify CDC survival after 7 days cell culture under ischemic conditions (Figure 2.11). For the control group without loaded bFGF, a significant cell death of CDCs was observed as only 25.17% of CDCs left after 7 days. However, in the bFGF loaded group, there was no significant cell number changing on Day 3 and on Day 7 compared with that of Day 1. The result demonstrated that the bFGF releasing hydrogel enhanced CDCs survival under ischemic conditions.

To study the expression of paracrine factors from the survived cells, real-time RT-PCR was used. Four paracrine factors, PDGF, HGF, IGF1 and VEGF, were examined at mRNA level. The result showed fold increase of all four paracrine factors of CDCs in bFGF loaded hydrogel comparing to the no-bFGF loaded control group (Figure 2.12). Among them, PDGF, VEGF and HGF increased significantly. These indicated that the survived CDCs could secrete paracrine factors. The upregulated paracrine factors helped stem cells survive in harsh ischemic culture conditions and promoted angiogenesis in the tissue as well.

Western blotting result (Figure 2.13) showed that CDCs in bFGF releasing hydrogel had higher expression of p-Erk1/2 which were two extracellular signal-regulated protein kinases. The higher expression of the p-Erk1/2 indicated that the pathway was activated by
stimuli via bFGF. As it is well known, this pathway controlled cell proliferation and apoptosis. From a mechanism point of view, CDCs survival and proliferation under ischemic conditions were enhanced in bFGF releasing hydrogel system.

2.3.5 CDC survival, proliferation and differentiation after injected into rat MI heart model with bFGF release system in vivo

To test the efficacy of bFGF releasing hydrogel system to promote CDCs survival in vivo, the live cell tracker labeled CDCs were mixed with hydrogel with and without addition of bFGF. The mixture hydrogel solution was injectable through a 26-gauge needle and was observed to have fast gelation in the injection site of heart tissue within 7 seconds. The CDCs in hydrogels with and without bFGF releasing were injected into the ischemic rat heart for 2 weeks.

To characterize the survival CDCs after injection, confocal images were taken with tissue sections on the slices. The CM-DiL labeled CDCs showed fluorescence at 546nm (Figure 2.14). The live cell number of CDCs in hydrogel loaded with bFGF was significantly increased. Besides, migration of survival cells from the hydrogel to the host tissue was observed from the images. To characterization the proliferation and differentiation of the implanted CDCs at protein level, the sections were stained with Ki67 and myosin heavy chain (MHC). Among the surviving injected CDCs and host tissue cells, cells which were Ki67 positive could be observed. This results indicated that the injected survived CDCs could proliferate and some host cells also had proliferation characteristics (Figure 2.15). In bFGF releasing system, more cells were observed to express the cardiac
marker than those cells in no-bFGF loaded hydrogel (Figure 2.16). Angiogenesis improvement was evaluated by the number of vWF positive tubular structures (Figure 2.17). More tubular structures were found in the bFGF releasing groups than that in the non-bFGF releasing system. This indicated that the angiogenesis was promoted by the injection of CDCs with angiogenesis growth factor bFGF releasing system.

2.4 Discussion

Stem cell therapy was applied to treat acute and chronic myocardial infarction. The injected stem cells were expected to proliferate and differentiate to regenerate the damaged heart tissue. However, two major issues associated with the simple stem cell injection method resulting low therapy efficacy: firstly, low cell retention rate and large factions of cells leaked out during or soon after injection due to the low viscosity of the cell suspension and motion of heart, and secondly low inferior cell engraftment rate. It was reported that less than 0.3% injected cells survived in a few weeks after injection. This was mainly because of the harsh ischemic environment (limited oxygen and nutrients supply) in the infarct heart tissue and lack of extracellular matrix led to cell apoptosis.

In this work, the objective was to develop a stem cell carrier that only deliver cells but also augment stem cell survival under ischemic conditions. An injectable thermosensitive hydrogel based bFGF delivery system was developed to address the above issues to improve the efficacy of stem cell therapy. The system was based on CDCs, a biodegradable thermosensitive hydrogel NIPAAm/APLA/HEMA, and bFGF. CDCs were able to differentiate into many cell types in cardiac tissues which would be a good option for
cardiac tissue regeneration. bFGF was a pro-survival and proangiogenic growth factor which could promote stem cell survival and stimulate blood vessel formation [139-142]. The injectable thermosensitive hydrogel served as a carrier for stem cell and bFGF delivery. It had higher viscosity as well as fast gelation time (less than 7s) which increased the cell retention rate when injection was made [54]. In addition, it provided the stem cells an ideal extracellular matrix to attach and a microenvironment for proliferation and differentiation. The hydrogel was slowly biodegradable with 6.7% weight loss after 8 weeks which allowed sufficient protection to the cells before the regeneration and vascularization was established. In the system, the encapsulated bFGF was able to gradually release from the hydrogel for at least 28 days (Figure 2.7). Besides, the released bFGF, though encapsulated for a long time, showed bioactivity which not only should enhance stem cell survival but also promote angiogenesis (Figure 2.8). The released bFGF from the hydrogel promoted angiogenesis which was demonstrated by the significant blood vessel quantity increase by subcutaneous injection of bFGF releasing hydrogel in vivo (Figure 2.9).

In the in vitro study, CDCs in the hydrogel without bFGF release died dramatically (more than 70%) in 7 days under ischemic conditions which mimicked the infarct heart environment. However, by encapsulating bFGF into the hydrogel, no significant cell death was observed after 7 days. It was reported previously that bFGF could inhibit cell apoptosis. Erk1/2 were two extracellular signal-regulated protein kinases and the pathway controlled cell proliferation and apoptosis. From western blotting study of cell proliferation and apoptosis pathway Erk1/2, the bFGF group showed high Erk1/2 expression which indicated that the pathway was activated in the bFGF releasing system (Figure 2.13). The
initial CDCs survival and proliferation could be a result of the activation of the pathway. In addition, the paracrine effect was confirmed at mRNA level that the survived CDCs were able to secret paracrine factors, including PDGF, HGF, IGF1 and VEGF. The paracrine growth factors could in turn enhance cell survival, proliferation, migration and stimulate angiogenesis as well [143]. It needed to be noticed that, among all the factors, VEGFA showed highest fold increase which indicated a high vascularization effect potential. Expression of PDGF was reported to active Erk pathway to enhance cell proliferation [144]. It could be deduced that the developed bFGF releasing system was able to promote stem cell survival under ischemic environment in vitro by activation of the proliferation and apoptosis pathway and upregulation of paracrine factors.

In the in vivo study, CDCs in hydrogel with and without bFGF were injected into the infarct rat heart tissue. The long term live cell tracker images demonstrated the survival of cells under ischemic environment in the infarct heart for both conditions. The survival of CDCs in infarct heart was significantly enhanced by the bFGF releasing system and the result was consistent with the in vitro results. Beside of the enhanced cell retention and engraftment, the migration of survived CDCs to the host tissue was also observed. This phenomenon was a result of degradation of hydrogel and the motion of cells along the surface of the hydrogel which was stimulated by the paracrine factors, like PDGF. The migration of stem cells allowed the connection between cells and host tissue which promoted better tissue regeneration. The number of Ki67 positive CDCs were observed significantly higher in injected CDCs and host cells in bFGF releasing group than that of the no-bFGF group. The bFGF not only enhanced stem cell survival under ischemic
conditions but also promoted stem cell and host cell proliferation [141, 145]. The higher CDCs and host cell proliferation could also be a result of paracrine effect and the migration of CDCs to the host tissue.

It was well known that bFGF stimulated angiogenesis [48]. In addition, VEGF, PDGF and HGF were proven to play critical role in angiogenesis. bFGF releasing system which enhanced the gene expression of the paracrine factors could also promote angiogenesis which was confirmed by the higher number of vWF positive tubular structure in the bFGF release system injection heart. The enhanced vascularization was attributed to the effect of released angiogenic growth factor bFGF from the hydrogel and the high upregulated paracrine factor VEGFA from the survived CDCs. The faster establishment of angiogenesis helped vascularization in the infarct heart tissue to restore blood reperfusion. Thus the oxygen and nutrients could be supplied to the ischemic environment and help regenerate the tissues.

In addition, bFGF releasing system could promote heart muscle regeneration. The protein expression of cardiac marker MHC was observed in the survived cells which indicated that the survived stem cells had the potential to differentiate and to regenerate the damaged cardiac tissue. The myogenic differentiation of CDCs was owing to the fact that the survived CDCs secret paracrine factors, such as IGF1 for promyogenesis and the released bFGF showed myogenic effect.
2.5 Conclusion

In this work, an injectable thermosensitive biodegradable hydrogel based bFGF release system was developed to improve stem cell survival and angiogenesis under ischemic conditions. The system was readily injectable and capable of releasing bFGF for 4 weeks while keeping bFGF bioactivity. In vitro results showed the survival of CDCs could be successfully enhanced when they were encapsulated in bFGF releasing hydrogel under ischemic conditions. In vivo results indicated that cell survival, engraftment with host tissues and proliferation were significantly improved when delivering CDCs in bFGF releasing hydrogel. Furthermore, angiogenesis and cell differentiation were promoted in the bFGF releasing hydrogel injected group. The aforementioned exciting improvements may result from significantly increased cell survival and integration proliferation, CDCs myogenic differentiation for heart tissue regeneration and endothelial differentiation for blood vessel formation, bFGF induced fast angiogenesis, and stimulated paracrine effect of the survived CDCs. The strategy of encapsulating bFGF in hydrogel for sustained release to enhance stem cell survival and angiogenesis for cardiac tissue regeneration provided a therapeutic approach to treat MI.
Table 2.1 Primer of paracrine factors for real time RT-PCR.

<table>
<thead>
<tr>
<th>Transcription</th>
<th>Primer sequences</th>
<th>Tm(°C)</th>
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| β-Actin       | Forward: AAGATCAAGATCATCTGGCTCCTC  
Reverse: GGACTCATCGTACTCCTG | 61.2   |
| PDGF          | Forward: GATGCCTTGGAGACAAACCTGACA  
Reverse: ATACTCTCTTCTCTCTGCAGATGGGC | 70.3   |
| IGF1          | Forward: TGACATGCCAAGACTGAGAGGA  
Reverse: GGATTGCTCAAGCAGAAAGATCT | 71.0   |
| HGF           | Forward: ATGATGTGGGAGCCCCCCACTTGCTG  
Reverse: GCAGAATTTGCTCCCCCACATCAT | 73.3   |
| VEGF          | Forward: GACATCTCTCCAGAGATACC  
Reverse: TGCTGTAGGAAGCTCATCT | 55.9   |


Figure 2.1 Synthesis scheme of NIPAAm-co-APLA-co-HEMA (86/4/10).
Figure 2.2 $^1$H-NMR spectrum of monomer APLA.
Figure 2.3 $^1$H-NMR spectrum of hydrogel copolymer NIPAAm-co-APLA-co-HEMA.
Figure 2.4 (a) Hydrogel solution was flowable at 4°C; (b) Hydrogel solution forms gel at 37°C; (c) Hydrogel solution can be injected through 26 gauge needle; (d) and (e) solid hydrogel was flexible.

Figure 2.5 Hydrogel degradation at 37°C in DPBS for 8 weeks.
Figure 2.6 Toxicity test of hydrogel degradation product.

Figure 2.7 bFGF releasing kinetics in 20% APLA hydrogel for 28 days.
Figure 2.8  Bioactivity of the released bFGF.
Figure 2.9 In vivo IHC staining of blood vessels for Gel and Gel/bFGF groups after 2 weeks subcutaneous injection.

Figure 2.10 Blood vessel density of Gel and Gel/bFGF after 2 weeks subcutaneous injection.
Figure 2.11 dsDNA content of Gel/CDCs, and Gel/bFGF/CDCs in ischemic conditions for 7 days. (*p<0.05, **p<0.01).

Figure 2.12 Paracrine effect (PDGFB, HGF, IGF1, and VEGFA) of Gel/CDC and Gel/CDC/bFGF culture in ischemic condition. β-actin was used as housekeeping gene (*p<0.05).
Figure 2.13 Western blotting result of p-Erk1/2 expression of Gel/CDC and Gel/CDC/bFGF culture in ischemic conditions for 7 days.
Figure 2.14 Cell survival of Gel/CDC and Gel/bFGF/CDC 2 weeks in vivo (A. Live cell images; B. Survived cell density. *p<0.05).
Figure 2.15 Ki67 expression of Gel/CDC and Gel/bFGF/CDC 2 weeks *in vivo* (A. Ki67 stained images; B. Ki67 positive cell density. *p<0.05).*
Figure 2.16 MHC expression of Gel/CDC and Gel/bFGF/CDC 2 weeks in vivo (A. MHC stained images; B. MHC positive cell density. *p<0.05).
Figure 2.17  Blood vessel formation of Gel/CDC and Gel/bFGF/CDC groups 2 weeks in vivo (A. vWF stained images; B. blood vessel density. *p<0.05).
CHAPTER 3: An injectable hydrogel with oxygen release to augment cardiosphere-derived cells and neural stem cells survival under ischemic conditions

3.1 Introduction

Heart and brain diseases, especially myocardial infarction (MI) and stroke are leading cause of morbidity and mortality worldwide [9, 146]. They are major cardiovascular diseases which caused massive cell death and partial loss of tissue function. When both diseases occur, a blockage of blood vessel results extremely limited blood supply to the tissue area. An ischemic environment (low oxygen and low nutrient) is formed in the infarct region which lead to tissue death. The necrotic tissue is then removed by macrophages and eventually a scar tissue is formed. Because the cardiomyocytes and neurons are unable to regenerate by themselves, the heart and brain function cannot be restored. In the end, the process leads to heart failure and brain damage.

Organ transplantation is a method to treat the disease but extremely limited by the number of donor organs and long term immune problems [147-149]. Development of a therapeutic method to repair the damaged tissue under ischemic conditions is a desirable strategy [150]. In such conditions, stem cell therapy is pursued as a potential approach to regenerate the damaged tissue and promote angiogenesis in the infarct tissue [9, 31, 121, 129, 137]. By injecting stem cells into the infarct region, the stem cells engraft in the damaged tissue area and differentiated into desired cell types (cardiomyocytes or neurons)
to replace the lost cells. The tissues are therefore regenerated and functions are restored afterward.

To investigate the possibility of implantation and the ability of regeneration, many types of cells were used for the therapy, including induced pluripotent stem cells (iPSCs), mesenchymal stem cells (MSCs), cardiac stem cells (CSCs), embryonic stem cells (ESCs), neural stem cells (NSCs) and cardiosphere-derived cells (CDCs) [20, 32, 125, 151-154]. Among these cells types, CDCs and NSCs are considered to be promising cell types for cardiac and neural regeneration. NSCs are able to differentiate into all cell types required in brain tissue regeneration [155, 156]. CDCs can be easily isolated from the explant of exocardium biopsies and have a high proliferation rate. A considerable number of CDCs can be acquired in relatively short culture period. CDCs are proved to be able to differentiate into cardiomyocytes in vitro and in vivo [157]. In addition, CDCs have a higher differentiation capability than MSCs. There is no ethical concerns and potential danger like ESCs.

However, it was reported that when injecting cells into heart, approximately 90% of cells were lost to the circulation or leak out of the injection site because of the heart motion [158]. For the cells retained in the injection site, most of the cells died within the first few weeks and only a small percentage of survived cells were observed to be able to differentiate [159]. Thus the efficacy of direct stem cell injection method is extremely low and the clinical application is limited. The causes of such a situation are mainly two aspects, cell carrier and ischemic environment [117]. The commonly used saline solution is very flowable and cannot hold the cells in the injection site. In the infarct heart, an extremely
low oxygen and low nutrient ischemic environment is generated and the transplanted cells can hardly survive in such harsh conditions [160-162].

A proper carrier plays a key role to increase the retention rate of cells after injection. In tissue engineering, biomaterials are widely used and investigated. An injectable biomaterial could be used to deliver cells into the infarct heart through syringe and needle. The injected biomaterials should help increase the cell retention and at same time provide cells with a microenvironment for cell attachment [39]. In addition, they can also be used to support infarct heart wall to avoid ventricular remodeling. Recently, different biomaterials were used in cardiac treatment for tissue regeneration, including natural materials such as collagen, chitosan, matrigel and fibrin, and synthesized materials such as PEGylated fibrinogen biomaterial, MPEG-PCL-MPEG hydrogel and poly (N-isopropylacrylamide) (PNIPAAm) based copolymers [142, 143, 163, 164].

To address the issue of cell survival under ischemic conditions, many approaches were reported. Ischemic preconditioning cells improved ischemic tolerance of cells in the infarct heart tissue [165, 166]. Genetic modulation of cells prior to transplantation enhanced cell viability and apoptotic resistance by delivering growth factors and anti-apoptotic factor genes into the cells [167, 168]. Heme oxygenase-1 (HO-1) plasmid modification of MSCs protected cells in 1% oxygen environment for 24 hours [169]. However, ischemic preconditioning may damage cells in the process and the implanted cells may not survive under ischemic conditions for enough long time which was need for cell differentiation and angiogenesis establishment. The operation of genetic modulation process was complicated and may bring safety concerns.
Introducing an oxygen release system which can release oxygen for a relatively long time at a considerably high level to the infarct tissue may be considered an ideal approach. Inorganic peroxides were used as oxygen producing substances. However, the calcium or sodium ions of the substances could affect the function of heart [85]. This inorganic peroxide base oxygen releasing system should not be used in heart injection. Hydroperoxide could convert to oxygen and water in the presence of catalase [89]. However, it was reported that the exposure of hydroperoxide to cells threatened the cell growth as hydroperoxide producing reactive oxygen species. In addition, the oxygen generation process of hydroperoxide was too fast to support cell consumption for a long time [170]. Previous studies from our group demonstrated that H$_2$O$_2$ mixed with poly (vinyl pyrrolidone) (PVP) could form a complex which stabilizes H$_2$O$_2$ [90, 171]. And core-shell structure microspheres could be fabricated with H$_2$O$_2$/PVP complex for the core and PLGA for the shell. The microspheres shell material PLGA is a FDA approved biodegradable material. When PLGA slowly degrades, the H$_2$O$_2$/PVP complex releases continuously from the microspheres and H$_2$O$_2$ converted to oxygen at the presence of catalase in the hydrogel system. The release of oxygen could last for at least 2 weeks.

To address the above two major limitations, a new biodegradable and thermosensitive hydrogel with relatively high oxygen permeability was designed and synthesized and oxygen release microspheres were made and improved by surface modification with catalase. We proposed the development of oxygen release system by combining the oxygen release microspheres and the hydrogel. The thermosensitive hydrogel was liquid in low temperature (4°C) and solidifies at 37°C quickly. It could be injected through a 26-gauge
needle. The hydrogel solution served as a carrier of stem cells and microspheres when delivering stem cells into infarct heart. The hydrogel solution had higher viscosity than saline and collagen gel. It solidified quickly at 37°C and had high oxygen permeability and flexibility. With enclosure of the oxygen release microspheres inside, the hydrogel not only holds the delivered stem cells in the injection site which increased the cell retention rate, but also provided stem cells with a sustained oxygen generating environment in the injected infarct region which enhanced injected stem cell survival, differentiation and angiogenesis under ischemic conditions. In this work, we studied how oxygen release system can affect CDCs survival under ischemic conditions \textit{in vitro} and \textit{in vivo}.

### 3.2 Materials and methods

#### 3.2.1 Materials

2-hydroxyethyl methacrylate (HEMA, Alfa Aesar) was purified by passing through a column loaded with inhibitor remover. N-isopropylacrylamide (NIPAAm, TCI) was recrystallized with hexane three times before use. Poly (lactide-co-glycolic acid) (PLGA, LACTEL) has a LA/GA ratio 50/50 and inherent viscosity 0.55-0.75. Hydrogen peroxide (30% aqueous solution, Sigma), bovine liver catalase (2000-5000 units/mg, Sigma), poly (N-vinylpyrrolidone) (PVP, Sigma) with molecular weight 40-90 kDa, HO-PEG3000-NH\textsubscript{2} (Jenkem), biotin hydrazide (Sigma), Ru (Ph\textsubscript{2}phen\textsubscript{3})Cl\textsubscript{2} (GFS Inc.), and rhodamine-b (Sigma) were used as received.
3.2.2 Synthesis of hydrogel polymer and PLA-PEG-Biotin

The hydrogel was synthesized by copolymerization of NIPAAm, APLA and HEMA, using free radical polymerization with a molar feeding ratio of 86/4/10 as described in Chapter 2 (Figure 2.1). NIPAAm, APLA and HEMA were dissolved in dioxane in a 250mL, three-neck round bottom flask. The initiator benzoyl peroxide (BPO) was added and the polymerization was conducted in 70°C oil bath with nitrogen protection overnight. The polymer was precipitated out by pouring the solution into hexane. Then the polymer was further purified twice by dissolving in tetrahydrofuran (THF) and precipitating in ethyl ether. The polymer was vacuum dried overnight before use. The structure and ratio of components were confirmed by $^1$H-NMR.

PLA-PEG-Biotin was synthesized following the reported method which included three steps [172]. 1) Synthesis biotin-NHS. Biotin (5g, 20.5mmol) was dissolved in 150mL of DMF at 60°C, and then treated with dicyclohexylcarbodiimide (DCC, 4.3g, 20.8mmol) and N-hydroxysuccinimide (NHS, 2.5g, 21.7mmol). The mixture was stirred at 60°C for 2h and kept stirring for 24h at room temperature with N$_2$ protection. The precipitate was filtered and the clear solution was saved for next step. A yield of 20% was obtained. The product biotin-NHS will be 0.14g (0.41mmol) in 15mL DMF. 2) Synthesis biotin-PEG-OH. HO-PEG3000-NH$_2$ (0.56g, 0.165mmol) was dissolved into acetonitrile (1.11mL, 0.0213mmol) followed by adding of methylene chloride (1mL) and triethylamine (0.322mmol, 45μL). The mixture was then stirred for 1min. After adding the solution of previous step (0.14g biotin-NHS in 15mL DMF), the reaction was stirred overnight under protection of nitrogen. The polymer was precipitated out by the slow addition of diethyl ether (40mL) to the
solution. The polymer was then filtered and washed with diethyl ether. The isolated polymer was dissolved in THF and centrifuged. The supernatant was poured into diethyl ether with stirring. The precipitate was filtered and vacuum dried overnight.

3) Synthesis of biotin-PEG-PLA. The round bottom flask was charged with lactide (2g, 0.0139mol) and biotin-PEG-OH (0.35g, 0.1mmol). The flask was sealed under nitrogen and heated at 140°C for 16h. This polymer melt was cooled down and dissolve in THF. The solution was then added dropwise to 100mL diethyl ether with stirring to obtain the precipitate polymer. The final product was isolated by vacuum filtration and freeze dried overnight. The structure was confirmed by 1H-NMR.

3.2.3 Characterization of properties of the hydrogel

Hydrogel solutions in this study were prepared by dissolving the hydrogel polymer in Dulbecco’s modified phosphate buffer saline (DPBS, pH = 7.4) at a concentration of 20% (w/v) (The concentration of hydrogel solution is 20% in the following content if no specific comment is made.). The thermal transition temperature of the solution was measured by using differential scanning calorimetry (DSC) over a temperature range of 0-60°C, with a heating rate of 10°C/min [130]. Injectability of the hydrogel solution was tested with a 26-gauge needle which is also used for injection in animal surgery [173]. The gelation time of the hydrogel solution at 37°C was measured using an Olympus IX771 microscope equipped with a temperature control chamber [54]. The hydrogel solution was dropped onto a pre-warmed glass slide on the microscope stage. The time was recorded when the transmittance of the hydrogel solution from clear to opaque was observed.
The solid hydrogel was acquired after the hydrogel solution was gelled in a 37°C water bath. After several hours to reach equilibrium water content, the hydrogel was ready for water content and mechanical test. The wet weight of the hydrogel was measured as $w_1$, while the lyophilized hydrogel weight was measured as $w_2$. The water content was calculated as:

$$\text{Water content (\%) = } \frac{w_1 - w_2}{w_2} \times 100\%$$

The mechanical properties of the hydrogel were tested with an Instron tensile tester, using a cross-head speed of 50 mm/min. The hydrogel modulus was calculated from the elastic deformation region of the stress-strain curves, using a MATLAB program.

To determine hydrogel degradation, microspheres were first mixed in hydrogel solution at a concentration of 50mg/mL. 200μL of hydrogel/microsphere mixture were added to a 1.5mL microcentrifuge tubes and the tubes were placed in a 37°C water bath. After incubation for several hours, the supernatant was replaced with 200μL DPBS. The degradation was conducted for 8 weeks at 37°C. The samples were taken at each time point and weight of hydrogel was measured as $w_3$ after they were freeze-dried. The weight of sample before degradation (week 0) was $w_4$. The weight remaining was calculated as:

$$\text{Weight remaining (\%) = } \frac{w_3}{w_4} \times 100\%$$

The degradation product toxicity was tested with MTT assay with degradation product DPBS solution at concentrations of 1mg/mL and 5mg/mL.
3.2.4 Preparation of oxygen releasing microspheres and catalase conjugation

The oxygen releasing microspheres were prepared by a co-axial electrospinning device (Figure 3.1) with PLGA or PLGA/PLA-PEG-Biotin (50/50) as shell and PVP/H₂O₂ as core. The syringe connected to core was charged with PVP and H₂O₂ complex with H₂O₂/VP ratio of 4.5/1 (10mL 30% H₂O₂, 2.42g PVP40000) and the syringe connected to shell was charged with 5wt% PLGA or PLGA/PLA-PEG-Biotin (50/50) solution (in Dichloromethane). The co-axial device was connected to a positive power supply at a voltage of +17kV and the collector was connected to a negative power supply at a voltage of -10kV. The infusion rates for the PVP/H₂O₂ complex and PLGA or PLGA/PLA-PEG-Biotin (50/50) solution were 0.2 and 1 mL/hr. Another syringe which was filled with PEG10000 solution (30wt% in DCM) and charged with +15kV was sprayed at an infusion rate of 17mL/hr. The microspheres which were dispersed in PEG were collected on a piece of aluminum foil. The solid on the foil was then dispersed in DI water and centrifuged at 11000rpm to obtain the microspheres. The process was repeated twice and the microspheres were freeze dried for 2h and stored in -20°C freezer before use. To characterize the core-shell structure of the microsphere, FITC (10mg/mL) and rhodamine-B (7mg/mL) were added to the solutions for core and shell, respectively. Confocal microscope was used to detect the two fluorescent dyes which indicated the structure.

To conjugate catalase on the oxygen releasing microspheres, PLGA/PLA-PEG-Biotin (50/50) were fabricated as shell of the microspheres and biotin-streptavidin conjugation method was used to modify the microspheres surface with catalase. The catalase was labeled with biotin by ProtOn™ Biotin Labeling Kit (Vector), following the protocol...
provided in the kit. To visualize catalase on the shell of the microspheres, catalase was labeled with FITC. 10mg/mL catalase solution was dialysis against 2L of 100mM sodium carbonate solution (pH=9.3) at 4°C for 24 hours. 4mg FITC was added into 1mL 100mM sodium carbonate (pH 9.3) to make a 100X FITC stock solution. To label FITC on the catalase, 25µL FITC stock solution was added to 1mL 10mg/mL catalase solution and the mixture was incubated at room temperature for 2 hours. The FITC labeled catalase was purified via dialysis in DI water at 4°C for 24 hours. The catalase solution was stored at 4°C in dark and used within 1 week. After the microspheres were stirred in 1mg/mL streptavidin solution at 4°C for 2 hours, the mixture was centrifuged at 10000rpm for 10min and the microspheres were washed twice with distilled water. Then the microspheres (50mg/mL) was stirred in 0.15mg/mL and 0.30mg/mL catalase-biotin solutions respectively at 4°C for 2 hours, again the mixture was centrifuged at 10000rpm for 10min and the microspheres were washed with distilled water twice. The microspheres were freeze dry for 2 hours and stored in -20°C. All processes which involved in using FITC were operated in dark. To determine the catalase conjugation density, the fluorescent intensities of the FITC labelled catalase-biotin solutions before and after conjugation were measured. The concentrations of the solutions were converted via an intensity-concentration calibration curve. The amount of conjugated catalase-biotin can be calculated from the concentration difference of the solutions before and after conjugation reaction.
3.2.5 Oxygen release kinetics.

The core-shell oxygen release microspheres with different H\textsubscript{2}O\textsubscript{2}/VP ratios were fabricated. Sustained oxygen releasing was conducted for 4 weeks by detecting intensity of oxygen sensitive fluorescent. To achieve this, an oxygen-sensitive luminophore Ru(\(\text{Ph}_2\text{phen}_3\))\textsubscript{Cl}_2 (7mg) and oxygen-insensitive fluorophore rhodamine-B (5mg) were mixed with polydimethylsiloxane (PDMS) precursor (10g) before the curing agent (1g) was added. The mixed was incubate in 50°C for half an hour to form a PDMS membrane which is highly oxygen permeable and hydrophobic. The membrane was cut to disks of 6mm diameter and placed into wells of a 96-well solid black microplate (Corning). After 200μL DPBS and 1mg/mL bovine catalase (for conditions of microspheres without catalase conjugation) were added to each well, the plate was incubated in 1% oxygen incubator overnight to balance the oxygen content. Then 10mg microspheres was quickly added to each well and the plate was sealed with transparent film (Thermal\textsuperscript{®}Seal films classic, polypropylene, Sigma). The oxygen release process was performed in 1% oxygen and 37°C incubator for two weeks. At Day1, 2, 3, 4, 5, 6, 7, 10 and 14, the plate was read by a fluorescent plate reader at 610 nm for Ru(\(\text{Ph}_2\text{phen}_3\))\textsubscript{Cl}_2 (470 nm excitation) and 576 nm for rhodamine-B (excitation at 543 nm). Standard oxygen concentration-fluorescent intensity calibration curve was made by reading fluorescent intensity of DPBS solution under 1, 5, 15, and 21% oxygen. Oxygen concentration of each time point was converted by the standard curve.
3.2.6 Cardiosphere-derived cells (CDCs) and neural stem cells (NSCs) culture.

Mouse CDCs were isolated from the atrium of the C57BL/6 mouse following previously established method [125]. The culture medium contained Iscove’s Modified Dulbecco’s Medium (IMDM, Invitrogen), 10% fetal bovine serum (FBS, Atlanta Biologicals), 1% Penicillin-streptomycin (Invitrogen). The cells were cultured under normal culture conditions (21% O₂, 5% CO₂, 37°C). The culture medium was changed every three days and CDCs were passaged when 90% confluence was reached. Cells at passage 11-15 were used for cell encapsulation into hydrogel. CDCs at these passages preserved phenotype and multipotency.

Enteric mouse neural stem cells (NSCs) were generated following the reported method [174, 175]. Time mated c57BL/6-Tg (pan-EGFP) mice (Jackson Laboratory) were sacrificed and intestines from 12.5-day post-coitum embryos were dissected into Dulbecco’s modified Eagle’s medium/Ham’s nutrient mixture F12 (DMEM/F12, Invitrogen). Mouse intestines were dissociated in 50μg/mL dispase and 50μg/mL collagenase (Worthington Biochemical) for 60 minutes at 37°C. Intestines were triturated and filtered through 40μm cell strainers to obtain single cell suspensions. The obtained cells were cultured in 35 mm Petri dishes in DMEM/F12 medium containing 100 U/ml penicillin and 100μg/ml streptomycin (Invitrogen), supplemented with 2mmol/L L-glutamine (Invitrogen), 7.5% (v/v) chick embryo extract (Gemini Bio-products), 1% (v/v) N2 medium supplement (Sigma–Aldrich), 20ng/mL mouse basic fibroblast growth factor, and 20ng/mL mouse epidermal growth factor (Sigma). Medium was changed every four days. NSCs grew as free-floating cellular aggregates known as neurosphere-like bodies.
Cell passage was proceeded at 90-95% confluence. Cells were dissociated into single cells and subculture again. Cells within passage 7 were used in this experiment to make sure the multipotency of the NSCs.

3.2.7 Encapsulation of oxygen releasing microspheres and cells into hydrogel.

The hydrogel solution was prepared by dissolving hydrogel polymer in DPBS at a concentration of 20%(w/v) on ice. The hydrogel solution was sterilized under UV for 30 minutes before use. Oxygen releasing microspheres and catalase were added into the hydrogel solution at a concentration of 50mg/mL and 1mg/mL, respectively. CDCs were detached from the cell culture plate by Trypsin-EDTA and suspended in small amount of DPBS and added into the hydrogel solution to a final concentration of 8 million/mL. NSCs were digested to single cells from neural stem cell spheres by Trypsin-EDTA and suspended in small amount of DPBS and added into the hydrogel solution to a final concentration of 4 million/mL. A 1mL pre-cooled syringe was used to pipette the mixture at least 20 times to make them thoroughly mixed. All the procedures above were operated on ice.

3.2.8 CDCs survival under ischemic conditions in hydrogel loaded with oxygen release microspheres and generation of paracrine factors.

0.2mL cells and hydrogel solution mixture was transferred into each 1.5mL microcentrifuge tube and the gelation was performed in 37°C water bath for 30 minutes.
After the gelation, the supernatant was replaced with 200uL culture medium without FBS and a hole was punched on top of the tube. The tubes were incubated in hypoxia incubator (1% O$_2$, 5%CO$_2$, 37°C) for 7 days and the culture medium was replaced every three days. After 1, 3 and 7 days of culture, the gels were digested by papain solution. Cell survival was quantified by dsDNA content (for live cells) which was measured by a Quant-iT™ PicoGreen dsDNA Assay Kit, following the provided protocol.

Gene expressions for paracrine factors were characterized by real-time RT-PCR. RNA was isolated from the gels using TRIzol following the protocol provided by Sigma. The quality of RNA was measured by NanoDrop and then used for cDNA synthesis with a High Capacity cDNA Reverse Transcription kit. Primers of forward and reverse pairs of the platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and insulin-like growth factor 1 (IGF1) were selected for CDCs gene expression study. β-actin was used as the housekeeping gene. The sequences, melting temperature, and expected product sizes are listed in Table 2.1. Real-time RT-PCR was performed three times for each sample with Maxima SYBR Green/Fluorescein master mix on an Applied Biosystems 7900 system. β-actin was used as the housekeeping gene. Fold increase was calculated using a standard ΔΔCt method.

Oxygen partial pressure in cells was determined by electron paramagnetic resonance (EPR) following the reported method [130, 176]. 0.1g EPR sensitive oxygen probe LiNc-BuO (OxySpin) was dispersed in 1mL 1% BSA solution and the mixture was sonicated on ice with 10s/cycle for 5cycles. 5μL probe mixture was added to 1mL cell culture medium. The plate was shaken gently every 2 hours in the first day. And the plate was incubated for
one more day to allow cells to swallow the probe. To the detachment of CDCs from the cell culture plate, the medium was first removed and CDCs were washed with DPBS 3 times. Then CDCs were detached from the plate by Trypsin-EDTA. The CDCs in hydrogel with and without oxygen releasing microspheres were added into EPR tubes at 4°C and gelation at 37°C. After adding no FBS culture medium, the EPR tube was transferred into the hypoxia incubator. After 3 days incubation, the EPR tubes were quickly sealed with a plastic cap and parafilm. Immediately after the tube was taken out and sealed, the oxygen partial pressure measurements were performed using an L-band EPR spectrometer (Magnettech, Berlin, Germany). EPR spectra were acquired as single 60s duration scans. The peak-to-peak width of the EPR spectrum was used to calculate oxygen partial pressure. The peak-to-peak width of the sample was recorded as $G$. A calibration was made by test the probe at room air and anoxia. To be specific, the probe was placed in a gas permeable capillary tube. A spectrum was scanned and peak-to-peak width was obtained ($G_0$) after the tube was flushed with 100% nitrogen for 17 min to remove any oxygen in the tube. Then tube was then flushed with 21% oxygen for 17min to get the peak-to-peak width ($G_1$) of the probe at room air condition. The oxygen partial pressure in room air is known as 160mmHg. The oxygen partial pressure in cell was therefore calculated:

$$P_{oxygen} = \frac{(G - G_0)}{(G_1 - G_0)} \times 160 \text{ mmHg}.$$
3.2.9 CDCs survival under ischemic conditions in hydrogel loaded with oxygen release microspheres and generation of paracrine factors.

0.2mL cells and hydrogel solution mixture was transferred into each 1.5mL microcentrifuge tube and the gelation was performed in 37°C water bath for 30 minutes. After the gelation, the supernatant was replaced with 200µL culture medium without FBS and a hole was punched on top of the tube. The tubes were incubated in hypoxia incubator (1% O₂, 5%CO₂, 37°C) for 14 days and the culture medium was replaced every three days. After 1, 7 and 14 days of culture, the gels were digested by papain solution. Cell survival was quantified by dsDNA content (for live cells) which was measured by a Quant-iT™ PicoGreen dsDNA Assay Kit, following the provided protocol.

Gene expressions for NSC differentiation were characterized by real-time RT-PCR. RNA was isolated from the gels using TRIzol following the protocol provided by Sigma. The quality of RNA was measured by NanoDrop and then used for cDNA synthesis with a High Capacity cDNA Reverse Transcription kit (ABI). Primers of forward and reverse pairs of the Glial fibrillary acidic protein (GFAP), NESTIN, and microtubule-associated protein 2 (MAP2) were selected for NSCs gene expression study. β-actin was used as the housekeeping gene. The primers were purchased from Sigma-Aldrich and the sequences, melting temperature, and expected product sizes are listed in Table 3.1. Real-time RT-PCR was performed three times for each sample with Maxima SYBR Green/Fluorescein master mix. β-actin was used as the housekeeping gene. Fold increase was calculated using a standard ΔΔCt method. For protein expression, the hydrogel samples were first fixed in 4%
paraformaldehyde for 1 h and then washed with PBS. The fixed samples were embedded in OCT (Tissue-Tek) and frozen sectioned at 5 µm. The sections were blocked by 10% goat serum blocking buffer with 0.3% Triton X-100 at 37°C for 1h. Immunohistological staining for GFAP, NESTIN, and MAP2 were performed on the tissue sections on the slides stained with primary antibody accordingly overnight at 37°C in a humid chamber. Secondary antibody was then conjugated to the primary antibody at room temperature for 1h. Nucleus were stained with DAPI. Negative control sections were prepared by the same procedure, but without the primary antibody incubation. The slides were observed and images were taken with FV2000 filter confocal microscope.

3.2.10 Injection of CDCs loaded oxygen release hydrogel into rat heart after acute MI

For in vivo study, acute MI model was made by ligating left coronary artery of rat heart. After 30 minutes, the system was injected through a 26-gauge needle into infarct area. CDCs were labeled with live cell tracker CM-DiL before injection. 0.2mL mixture hydrogel solution with CDCs concentration of 8 million/mL and microsphere concentration of 50mg/mL was injected into the infarct area at 3 different injection sites in the center of the infarct. The rat hearts were harvested after 2 weeks of implantation. The hearts were washed with DPBS three times and fixed with 4% paraformaldehyde at 37°C for 2 hours. The fixed hearts were cross sectioned at the infarct area and sent for paraffin embedding and sectioning at 5µm.
For immunohistochemistry, the sections were first stained with anti von Willebrand factor (vWF), anti Ki67 and anti-myosin heavy chain (MHC) primary antibody and then with corresponding fluorescent secondary antibody. Nuclei were stained with DAPI. The slices were imaged using an Olympus FV1000 confocal microscope. Angiogenesis and cell differentiation in the infarction tissue area was evaluated from the images. The sectioned slices were stained with vWF. Blood vessels were recognized as tubular structures which were vWF positively. Live cell, Ki67 and MHC positive cell numbers were calculated based on at least five different images. The left ventricle wall thickness of the infarct heart was calculated based on at least 5 sites on images of hematoxylin and eosin (H&E) stained slices.

3.2.11 Statistical methods

Data are expressed as mean ± standard deviation. Statistical comparisons were performed with a One-way ANOVA by JMP. Significant difference is defined as p < 0.05.

3.3. Results

3.3.1. Hydrogel and PLA-PEG-Biotin synthesis and hydrogel properties

The hydrogel polymer was synthesized by copolymerization of NIPAAm, APLA and HEMA using free radical polymerization method with a feed ratio of 86/4/10 as demonstrated in Chapter 2. PLA-PEG-Biotin was synthesized with biotin-NHS, HO-PEG-NH2 and lactide via three step process. The structures of the synthesized hydrogel
polymer and PLA-PEG-Biotin were confirmed by $^1$H-NMR spectrum (Figure 3.2). The molecular ratio of NIPAAm/APLA/HEMA was 86/4.2/9.8 which was consistent with the feed ratio.

The 20\% (w/v) APLA hydrogel solution was in liquid state and flowable at 4°C and was able to inject through a 26-gauge needle which is usually used for cells and materials injection into infarct heart (Figure 3.3). As described in Chapter 2, the hydrogel solution had a gelation temperature of 26.5 ± 0.2°C and was able to solidify under 37°C in 7 second. The short gelation time help ensure the gel retain in the heart after injection. After equilibrium the gel solution in 37°C, the soft gel had a water content of 48.7 ± 4.17 %. It showed highly flexibility with a Young’s modulus of 35 ± 5 kPa. After the oxygen releasing microspheres were added into the gel solution at a concentration of 50mg/mL, the mixture was allowed gelation at 37°C. The solid gel/microsphere system was incubated at 37°C in DPBS, it degraded slowly and had a weight remaining of 84.14% after 8 weeks (Figure 3.4). The degradation product was soluble at 37°C and not toxic to cells (Figure 3.5).

3.3.2. Fabrication of oxygen releasing microspheres and oxygen release kinetics

The oxygen release microspheres were prepared with a co-axial device using electrospray technique. By controlling the applied voltages of the electrical field and infusion rate of H$_2$O$_2$/PVP complex and PLGA solution or PLGA/PLA-PEG-Biotin (50/50), the core-shell structured microspheres were fabricated from the device. Different H$_2$O$_2$/VP microspheres were prepared to investigate different oxygen generation. The SEM
image of microspheres and the core-shell structure was showed by confocal images of the microspheres of which the core and shell are labeled with different fluorescent dyes (Figure 3.6). The conjugation of catalase on the surface of microspheres was based on biotin-streptavidin interaction with two different catalase concentrations. By labeling the catalase with fluorescent dye FITC, the catalase conjugation on the surface of microspheres was confirmed by the confocal image (Figure 3.7). And the catalase conjugation densities on the microspheres were able to be calculated by examining the fluorescent intensity. They were 7.33mg/g and 9.58mg/g respectively.

The releasing of oxygen was realized because H₂O₂ was slowly and continuously released from the microspheres when the shell PLGA degraded. Due to the complex form of H₂O₂ and PVP, the releasing of H₂O₂ can last for a relatively long period of time. The released H₂O₂ was converted to oxygen by catalase. The oxygen release kinetic was measured by converting fluorescent intensity of oxygen-sensitive luminophore referring to oxygen-insensitive fluorophore to oxygen concentration with a standard curve. The oxygen release curves showed microspheres with three different H₂O₂/VP ratio can sustainably release oxygen in two weeks (Figure 3.8). In the beginning, the oxygen released slowly. From Day 4 to 7, quick release was observed and highest point was reached at around Day 7. Microspheres with H₂O₂/VP ratio of 6/1 released the highest level of oxygen and remained at around 30% after Day 5. While the microspheres with H₂O₂/VP ratio of 4.5/1 showed very similar release curve for the first three days and reached highest oxygen level of 29.8% at Day 5. The oxygen level remained above 20% afterwards. The oxygen level of microspheres with H₂O₂/VP ratio of 3/1 steadily increased to 21% at Day 7 and then
dropped to around 10% in the following period of time. The releasing kinetics result indicated that microspheres with higher H₂O₂/VP ratio can release more oxygen and the oxygen level of microspheres with H₂O₂/VP ratio of 4.5/1 were much similar to the oxygen in the normal environment and were favored respectively. Catalase conjugation oxygen releasing microspheres had a H₂O₂/VP ratio of 4.5/1. The amount of catalase conjugated on the microspheres were calculated by the concentration difference of the catalase-biotin solution before and after the conjugation reaction. The releasing kinetics of microspheres with two catalase conjugation densities were studied. Both of the microspheres demonstrated continuously increasing oxygen level during the releasing time (Figure 3.9). The microspheres with catalase conjugation density of 7.33mg/g went above 10% after Day 4 and gradually increased to the maximum 23.5% at Day 14 while the microspheres with catalase conjugation density of 9.58mg/g raised above 15% after Day 4 and reached 31.1% at Day 14. This indicated that more oxygen was released by the microspheres with larger catalase conjugation density.

3.3.3. CDC survival in hydrogels with oxygen release under ischemic condition in vitro

Efficacy of oxygen releasing system was evaluated by culturing CDCs in the oxygen releasing system in vitro. CDCs were mixed with hydrogel solution with addition of oxygen releasing microspheres with H₂O₂/VP ratio of 4.5/1 and cultured in a hypoxic incubator (1%O₂, 5%CO₂, 37 °C) and supplied with no FBS culture medium which mimicked the ischemic environment in the infarct heart. The gelation time at 37 °C remained to be around 7 seconds.
The dsDNA content was measured to quantify CDC survival after 7 days culture under ischemic conditions (Figure 3.10). For the control group without adding oxygen release microspheres, the CDCs died dramatically. After 7 days, only 25.2% of CDCs survived compared to the cells number of Day 1. However, in the normal oxygen release system, there is no significant cell death observed on Day 3 and cell number was almost doubled on Day 7. In the catalase modified oxygen release system, a solid growth of the cell number was observed. Compared to the cell number on Day 1, the number increased to 132.6% on Day 3 and 169.3% on Day 7.

To demonstrate the effect of the oxygen release microspheres on the cell survival under ischemic condition, oxygen partial pressure in the cells was measured with oxygen sensitive EPR prob. The oxygen partial pressure was 2.6mmHg in CDCs in non-oxygen releasing system while 23.8mmHg in CDCs in oxygen releasing system. The released oxygen in the oxygen releasing system can be taken and consumed by the cells.

To study the expression of paracrine factors from the survived cells, real-time RT-PCR was used for evaluation. Four paracrine factors, PDGF, HGF, IGF1 and VEGF, were examined at mRNA level. The result showed upregulated gene expression of all four paracrine factors of CDCs in oxygen releasing system with and without catalase conjugation compared to the non-oxygen releasing control group (Figure 3.11). Among them, PDGF and HGF increased significantly. These indicated that the survived CDCs were able to secrete paracrine factors which in turn enhanced cell survival and promoted angiogenesis. Interestingly, higher gene expression of PDGF, HGF and VEGF were observed in catalase modified groups than those of the normal oxygen release groups.
Western blotting result (Figure 3.12) showed that CDCs in hydrogel with oxygen releasing microspheres had higher expression of p-Erk1/2 which were two extracellular signal-regulated protein kinases. The higher expression of the p-Erk1/2 indicated that the Erk pathway was activated and this pathway was known to control cell proliferation and apoptosis. CDCs survival and proliferation under ischemic conditions were enhanced in oxygen releasing hydrogel system.

3.3.4 NSCs survival in hydrogels with oxygen release microspheres under ischemic condition in vitro

NSCs were cultured in hydrogel with oxygen releasing system to investigate the cell survival under ischemic conditions in vitro. NSCs were encapsulated in hydrogel with addition of oxygen releasing microspheres with H2O2/VP ratio of 4.5/1 and cultured in a hypoxic incubator (1% O2, 5%CO2, 37°C) and supplied with medium which contained no FBS. The gelation time at 37°C remained to be around 7 seconds.

NSCs survival was evaluated and quantified by measuring the dsDNA content of NSCs at Day 1, 7 and 14 (Figure 3.13A). For the control group without adding oxygen releasing microspheres, the NSCs died significantly. At Day 7 and Day 14, only 64.9% and 24.9% of NSCs were alive compared to the cells number of Day 1. However, in the normal oxygen release system, even though the NSCs still died during the culture period, less cells died during the culture period. At Day 14, 57.5% NSCs survived in the harsh conditions. The number of survived NSCs was more than 2 times that of the NSC/Gel group. Live cell images (Figure 3.13B) were consistent with the dsDNA results.
To study the differentiation ability of the survived cells, three neural gene markers (GFAP, NESTIN, and MAP2) were examined by real-time RT-PCR to quantify the differentiation at gene level. The result showed significant fold increase of 17, 151, and 78 for GFAP, NESTIN, and MAP2 respectively in oxygen releasing system while only 5, 76, and 11 of the non-oxygen releasing control group (Figure 3.14). For the immunohistochemistry staining images (Figure 3.15), the NSC/ORM group demonstrated protein expression of differentiation markers and consistent with gene expression data. These indicated that the survived NSCs were able to differentiate which should enhance tissue regeneration.

3.3.5 CDC survival after injected into rat MI heart model with oxygen release system in vivo

To test the efficacy of oxygen releasing system in augmenting CDCs survival in vivo, the live cell tracker labeled CDCs were mixed with hydrogel with and without addition of oxygen releasing microspheres. The mixture hydrogel solution was still injectable through a 26-gauge needle and fast gelation in the heart tissue within 7 seconds. The CDCs in hydrogels with and without oxygen releasing microspheres were injected into the ischemic rat heart for 2 weeks.

To characterize the survived CDCs after implantation, confocal images were taken on the slices. The CM-DiL labeled CDCs showed fluorescence at 546nm (Figure 3.18). The live cell number of CDCs in hydrogel with oxygen microspheres was significantly increased. The migration of survived cells from the hydrogel to the host tissue was
observed from the images. To characterize the proliferation and differentiation of the implanted CDCs at protein level, the sections were stained with Ki67 and myosin heavy chain (MHC). Among the survived implanted CDCs and host tissue cells, partial cells were Ki67 positive which indicated the cells had proliferation characteristics (Figure 3.19). In oxygen releasing system, larger numbers of cells were observed to express the cardiac marker (Figure 3.20). Angiogenesis improvement was evaluated by the number of vWF positive tubular structures (Figure 3.21). Some survived CDCs were vWF positive which indicated that the CDCs were able to differentiate into endothelial cells to help develop blood vessels. More tubular structures were found in the oxygen releasing groups than that in the non-oxygen releasing system. This indicated that the angiogenesis was promoted by the injection of CDCs with oxygen releasing system.

3.4 Discussion

To overcome the harsh ischemic environment in the myocardial infarct area and improve the efficacy of the stem cell therapy is critical to treat MI. In this work, the developed injectable oxygen releasing system showed clinical potential to augment CDCs survival and enhance CDCs proliferation under ischemic conditions in vitro and in vivo. Basically, the injectable oxygen releasing system included an injectable thermal sensitive hydrogel and H₂O₂/PVP encapsulated oxygen releasing microspheres.

The NIPAAm based thermal sensitive hydrogel was developed to acquire a series of properties which were favorable for clinical injection. The hydrogel solution had a gelation temperature of around 26.5°C and fast gelation rate of around 7 second. The operations of
mixing the stem cells and hydrogel solution and injecting the mixture into the infarct heart tissue at 4 ℃ were feasible. The fast gelation rate enabled high retention rate of cells in the heart tissue after injection [177]. The hydrogel had a modulus a little lower than that of the heart tissue and was flexible which means the injected hydrogel will not affect the motion of heart. The uptake of oxygen of the cells in the hydrogel reflected that the hydrogel had a good oxygen permeability which meant that the oxygen can diffuse freely in the hydrogel and the encapsulated cells could take the released oxygen.

The very basic core-shell structure oxygen release microspheres were developed based on a previously reported method of our lab. These microspheres performed sustained oxygen release for at least two weeks. The generation of oxygen was due to the diffuse of the complex PVP/H2O2 while the PLGA degraded [178]. Three different H2O2 and VP ratio microspheres were fabricated and the oxygen release curves were made. Among them, microspheres with H2O2/VP ratio of 4.5/1 was picked due to the steady oxygen release level of just above 20%, which is closed to the oxygen level in the normal environment, from Day 4 to the end of release period. However, catalase was required to add into the hydrogel to convert the released H2O2 to oxygen. This reaction was conducted when the H2O2 met with the catalase. Therefore, there was a concern that the time gap between the H2O2 released out of the microspheres and met with catalase may postponed the oxygen formation and lowered the oxygen releasing efficiency. Modification of the shell surface of the microspheres by conjugating catalase on them was a feasible method to improve the oxygen conversion efficiency and the ease of operation. Biotin-streptavidin conjugation strategy was a fast and efficient conjugation technique to realize fast conjugation of biotin
labeled catalase on the microspheres with biotin functional groups on the surface via the strong interaction of streptavidin. Microspheres with two catalase conjugation density (7.33 and 9.58mg/g) were fabricated. The oxygen releasing kinetics were measured and the releasing curves were plotted. Both catalase modified oxygen releasing microspheres showed a continuously increased oxygen level for a two-week period of time. The release kinetics were related with the amount of catalase on the surface of the microspheres. The microspheres with catalase density 7.33mg/g was able to release oxygen to 12% at Day 5 and slowly to 23% after two weeks. For the microspheres with catalase density 9.58mg/g reached oxygen level of 12% a day ahead and the oxygen level went to 31% on Day 14. With more catalase conjugated on the surface of microspheres, more oxygen was produced at the same time point. This was because with more catalase conjugated on the microspheres surface, the microspheres became more hydrophilic and the PLGA degraded faster. Therefore more complex released from the microspheres. Aside from the difference of the releasing kinetics, there was a similar trend in both curves. Two small burst of oxygen release were observed at Day 2 and Day 4. According to the previous study, the H$_2$O$_2$/PVP solution trapped in the shell diffused out in the first two days. After Day 3, the diffusion of H$_2$O$_2$/PVP solution from core contributed to the second burst and the following sustained oxygen release.

In the *in vitro* study, CDCs in the hydrogel without oxygen release microspheres experienced dramatically death (more than 70%) after 7 days under ischemic conditions which mimicked the infarct heart environment. With the addition of oxygen release microspheres with and without catalase conjugation, no cell death was observed after 7
days. In contrast, the number of CDCs was increased to 169% and 182% for both kinds of microspheres. Since the oxygen level were 9% and 10% at day 3 and 19% and 25% at Day 7 for microspheres with and without catalase conjugation, it indicated that the oxygen at around 9% would be sufficient to maintain CDCs survival and more oxygen release afterwards may lead to increased cell number growth. The result of the oxygen partial pressure in CDCs demonstrated that it was the elevated oxygen level in the cells that enhanced cell survival. The survived CDCs were able to secret paracrine factors, including PDGF, HGF, IGF1 and VEGF. This was confirmed at the mRNA level (Figure 3.14). The paracrine growth factors could in turn enhance cell survival, proliferation, migration and stimulate angiogenesis as well. The High expression of Erk1/2 in Western blotting indicated that the pathway was activated in the oxygen releasing system. Erk1/2 were two extracellular signal-regulated protein kinases and the pathway controlled cell proliferation and apoptosis. From mechanism point of view, CDCs survival and proliferation could be a result of the activation of the pathway. Both cell survival results proven that the developed oxygen releasing system was able to promote stem cell survival under ischemic environment in vitro. Even though the oxygen releasing system was unlikely to promote NSCs proliferation, massive cell death under ischemic condition was suppressed with the oxygen supply from the microspheres. The survival rate of NSCs after 14 days ischemic cell culture was raised more than 2 times in oxygen releasing system from 24.9% to 57.5%. The survived NSCs had potential to differentiate into neural cell types. It was reported that the reduced oxygen restricted differentiation of many cell types [179-182]. From the gene expression and protein expression results, cell differentiation was largely limited without
oxygen. In Gel/NSC/ORM group, cell differentiation was observed. The expression of differentiation markers was tightly related to the supply of oxygen which enhanced cell survival and stimulated differentiation [183, 184].

In the *in vivo* study, CDCs in hydrogel with and without oxygen release microspheres were injected into the infarct rat heart tissue and the heart was taken, fixed and sectioned. The injection area tissue sections were examined. The long term live cell tracker images demonstrated the survival of cells under ischemic environment in the infarct heart for both conditions. The survival of cells may reflect that the fast gelation hydrogel improved the retention rate of injected cells. Since the oxygen can be released from the system for at least two weeks at a considerably high level of around 20% and the cells were able to take the benefit of the oxygen, more CDCs survived with addition of oxygen releasing microspheres compared with the CDCs implanted without oxygen releasing microspheres. The migration of survived CDCs to the host tissue was a result of degradation of hydrogel and the motion of cells along the surface of the hydrogel which was stimulated by the paracrine factors, like PDGF. The expression of proliferation marker Ki67 and cardiac marker MHC was observed among the survived cells. This indicated that the survived stem cells had the potential to proliferate and differentiate to regenerate the damaged cardiac tissue [127].

Due to the secretion of paracrine factors like VEGF, PDGF and HGF, angiogenesis was promoted. The above three growth factors were proven to play critical role in promoting angiogenesis. Oxygen releasing system which enhanced the gene expression of the paracrine factors could also promote angiogenesis which was confirmed by the higher
number of vWF positive tubular structure in the oxygen release system injection heart. The faster establishment of angiogenesis helped restore blood reperfusion to supply cell with oxygen and nutrients under ischemic environment and thus promoted tissue regeneration.

The left ventricle chamber dilates after MI. The injected hydrogel provided mechanical support to the infarct heart wall. Oxygen released from the system enhanced stem cells survival and proliferation which promoted the regeneration of damaged tissue and angiogenesis establishment. With the restoration of the function of heart tissue, the pace of dilating the left ventricle chamber slows down.

3.5 Conclusions

In this work, an injectable hydrogel based oxygen release system was developed to improve cell survival under ischemic conditions \textit{in vitro} and \textit{in vivo}. The hydrogel is thermosensitive and biodegradable. The system was injectable and capable of sustained releasing oxygen at certain level to support cell consumption for at least 2 weeks. Two type of stem cells, say CDCs and NSCs which were commonly used in stem cell therapy, were investigated in the developed hydrogel based oxygen releasing system. NSCs survival under ischemic culture in the developed oxygen releasing system was enhanced \textit{in vitro}. The survived NSCs demonstrated the ability to differentiate into neural cells at gene and protein level. CDCs survival was also significantly augmented \textit{in vitro} and \textit{in vivo} under ischemic conditions. The survival and proliferation of the stem cells were resulted from the released oxygen and stimulated paracrine effect which helped the cells overcome the harsh ischemic environment by supplying cells with oxygen and activation of proliferation.
pathway. In addition, by injecting CDCs with oxygen releasing system into infarct area of rat MI heart, promoted proliferation and differentiation of CDCs and angiogenesis in cardiac tissue were observed. This oxygen releasing system provided a potential therapeutic approach to address the problem of low cell survival when stem cell therapy was applied to regenerate the ischemia damaged tissue.
Table 3.1 Primers of neural differentiation genes for real time RT-PCR.

<table>
<thead>
<tr>
<th>Transcription</th>
<th>Primer sequences</th>
<th>Tm(℃)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Actin</td>
<td>Forward: AAGATCAAGATCATTGCTCCTC</td>
<td>61.2</td>
</tr>
<tr>
<td></td>
<td>Reverse: GGACTCATCGTACTCCTG</td>
<td>55.3</td>
</tr>
<tr>
<td>GFAP</td>
<td>Forward: TGGCTCGTGGATTTTGAG</td>
<td>68.5</td>
</tr>
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CHAPTER 4: Injectable thermosensitive hydrogel and hypericin based imagable oxygen releasing system to augment mesenchymal stem cells survival under ischemic conditions

4.1 Introduction

Ischemic condition refers to an extremely low oxygen and nutrition conditions in tissues [185]. This situation is mainly a result of vascular disease, such as vasoconstriction, embolism or thrombosis, and sometimes caused by trauma [186-188]. Cardiac ischemia, brain ischemia and limb ischemia are major ischemia disease types [189-192]. Usually ischemia appears when arteries is blocked and blood supply is restricted to the tissues. When ischemia occurs in tissues, there is insufficient of oxygen and inadequate of nutrients supplied to tissues which lead to tissue necrosis in a short time. Besides, limited blood supply also results reduced metabolic rate in tissues and cells die slowly under low metabolism [193]. For cardiac and brain tissue, ischemia causes irreversible damage in few minutes because these tissues are high aerobic tissues and very sensitive to hypoxia (low oxygen).

To address the shortage of oxygen issue, a direct way is to supply oxygen to the tissue under ischemic conditions. A quick intervention of oxygen supply to ischemia could prevent further damage to tissues and loss of tissue functions [194-197]. And long term of continuously oxygen supply could help tissue functioning normally and damaged tissue
regeneration before regular blood perfusion was established. An oxygen releasing system was successfully developed as introduced in Chapter 3. The microspheres based H$_2$O$_2$ releasing system could release oxygen at a considerably high level and the release sustained for relatively long time. Combined with stem cell therapy for cardiac and ischemic limb regeneration, hydrogel based oxygen releasing system significantly increased stem cell survival rate under ischemic conditions. Mesenchymal stem cells (MSCs) are multipotent stem cells that are able to differentiate into a variety of cell types, including osteoblast (bone cells), chondrocytes (cartilage cells), myocytes (muscle cells), and adipocytes (fat cells) [198-200]. The advantage of differentiation ability attributed to MSCs makes them typical stem cell options for tissue regeneration of stem cell therapy.

Beyond the oxygen releasing system fabricated in our group, many other oxygen releasing systems were also developed [85, 86, 201]. However, after the releasing systems are implanted in the required tissue, there is no way to detect the implant *in vivo* and the releasing conditions cannot be tracked or monitored in real time. To further make the oxygen releasing system more functional and applicable in clinical trials, a visible oxygen releasing system is in demand.

*In vivo* imaging technology enabled visualization and tracking of activities within a living organ, in real time by detecting fluorescent light from the fluorescent tagged materials, cells or tissues [202, 203]. Various fluorescent probes were reported for tumor detection *in vivo* [204]. This kind of technique made it a fundamental of the imagable oxygen releasing system. Hypericin (HYP) which was found in past decades and extracted from plants of genus Hypericum was regarded as a natural photosensitizing polycyclic
aromatic quinone [205, 206]. HYP was generally reported as a non-porphyrin photodynamic drug [207]. In photodynamic therapy (PDT) of cancer, it was recognized as an antiviral, antibacterial, and antineoplastic agent upon visible light irradiation [208-210]. Besides, HYP was also proposed as a fluorescent agent for photodiagnosis [211]. It was reported that by conjugating polyvinylpyrroidone (PVP), the hydrophobic molecule HYP could dissolve in water [212, 213]. The complex was found to be high photostable at low light irradiation and possessed a significantly enhanced fluorescent emission. It was reported that HYP (in DMSO) was excited at 555nm and had an emission at around 605nm [214]. This allowed the promising application of the HYP-PVP complex in photoimaging and photodiagnosis in vivo [215].

Based on our previous study of PVP/H$_2$O$_2$ complex which was used as core content in core-shell structured oxygen releasing microspheres [90], we hypothesized that HYP could be conjugated with PVP in H$_2$O$_2$ to form a stable complex which emit enhanced fluorescence. Therefore, the new complex should have potential to be used as core content in microspheres which was able to perform oxygen release as usual. Meanwhile by encapsulating the HYP/PVP/H$_2$O$_2$ complex into degradable polymer PLGA to form a core-shell structured microspheres, the microspheres should be fluorescent imagable and released oxygen for a long period of time in vivo [178]. Moreover, if possible, the release HYP which still maintain activity might be used to treat disease like cancer.

In this work, an injectable thermosensitive hydrogel based imagable oxygen releasing system was developed. The hydrogel served as carrier for the imagable oxygen releasing microspheres as well as cells. The oxygen releasing system was expected to provide
sufficient oxygen in a long time for cell survival and proliferation under ischemic conditions. By adding hypericin into the releasing complex to make a new three components complex which was used as the core material of the microspheres, the position of the injected oxygen releasing microspheres should be visible and the releasing process could also be monitored by in vivo imaging platform.

4.2 Materials and methods

4.2.1 Materials

N-isopropylacrylamide (NIPAAm, TCI) was recrystallized with hexane three times before use. 2-hydroxyethyl methacrylate (HEMA, Alfa Aesar) was purified by passing through a column loaded with inhibitor remover. Poly (lactide-co-glycolic acid) (PLGA, LACTEL) has a LA/GA ratio 50/50 and inherent viscosity 0.55-0.75. Hydrogen peroxide (30% aqueous solution, Sigma), bovine liver catalase (2000-5000 units/mg, Sigma), poly (N-vinylpyrrolidone) (PVP, Sigma) with molecular weight 40-90 kDa, hypericin (Alfa Aesar), Ru (Ph2phen3)Cl2 (GFS Inc.), and rhodamine were used as received.

4.2.2 Hydrogel polymer synthesis

Monomer APLA was synthesized following a two-step method as described in Chapter 2. The hydrogel polymer was copolymerized by using free radical polymerization method with monomers of NIPAAm, APLA, and HEMA at a molar feeding ratio of 86/4/10. In brief, a 250mL, three-neck round bottom flask was charged with NIPAAm,
APLA, and HEMA monomers. 120mL solvent dioxane was added into the flask. After the monomers were fully dissolved with stirring, initiator benzoyl peroxide (BPO) was added and the polymerization reaction was proceeded in 70°C oil bath overnight with nitrogen protection and magnetic stirring. The reaction was stopped and then the solution in the flask was poured into cold hexane with vigorous stirring to acquire the polymer precipitate. Then the polymer was vacuum filtered and was further purified twice by dissolving in tetrahydrofuran (THF) and precipitating in dry ethyl ether. The polymer was allowed vacuum dried for 24 hours before use. The structure of the hydrogel polymer and ratio of components were confirmed by $^1$H-NMR.

4.2.3 Hypericin/PVP/H$_2$O$_2$ complex preparation and characterization

2.5mL H$_2$O$_2$ and 0.6g PVP (M.W.=40000) were stirred at 4°C to form a complex of which the molar ratio of VP/H$_2$O$_2$ is 1/4.5. Hypericin (HYP) was then added to the complex according to the amount of PVP. To be specific, 3 kinds of complexes were made, HYP/PVP=15mg/1g, 10mg/1g, and 5mg/1g. The mixture was stirred under 4°C overnight and the prepared complex was kept avoid light in -20°C freezer.

To determine the fluorescent spectrum of the HYP/PVP/H$_2$O$_2$ complex, 2.5mL complex solution was transferred to a quartz cuvette and placed in UV-vis detect machine. The complex was scanned from wavelength 570 - 800nm with excitation of 555nm to obtain the emission intensity spectrum.
4.2.4 Fabrication imagable oxygen releasing microspheres

The oxygen releasing microspheres were prepared by a co-axial electrospinning device with PLGA as shell material and HYP/PVP/H$_2$O$_2$ as core (Figure 4.1). The syringe connected to inner needle was charged with HYP/PVP/H$_2$O$_2$ complex and the syringe connected to outer needle was charged with 5wt% PLGA solution (in Dichloromethane). The co-axial device was connected to a positive power supply at a voltage of +17kV and the collector was connected to a negative power supply at a voltage of -10kV. The infusion rates for the HYP/PVP/H$_2$O$_2$ complex and PLGA solution were 0.2 and 1 mL/hr. Another syringe which was filled with 30% PEG10000 solution (in DCM) and charged with +15kV was sprayed at a rate of 17mL/hr. The microspheres which were dispersed in PEG were collected on a piece of aluminum foil. The solid on the foil was then washed with DI water and centrifuged at 11000rpm to obtain the microspheres. The microspheres were washed with DI water twice and freeze dried for 2h before use. To characterize the core-shell structure of the microsphere, rhodamine-B was added to the solution for shell fabrication. Confocal microscope was used to detect the fluorescent of rhodamine-B and HYP which indicated the structure.

4.2.5 Oxygen releasing kinetics

The core-shell oxygen release microspheres with different HYP amount were fabricated and carefully stored. Sustained oxygen releasing was conducted for 4 weeks by detecting the intensity of oxygen sensitive fluorescent dye. To achieve this, an oxygen-sensitive PDMS membrane was made with luminophore Ru(Ph$_2$phen$_3$)Cl$_2$ and oxygen-
insensitive fluorophore rhodamine-B as described in previous chapter. The membrane was cut to disks of 6mm diameter and placed into wells of a 96-well black plate. After 200μL DPBS and 1mg/mL bovine catalase were added to each well, the plate was incubated in 1% oxygen incubator overnight to balance the oxygen content. Then 10mg microspheres was quickly added to each well and the plate was sealed with transparent film. The oxygen release process was performed in 1% oxygen and 37°C incubator for 4 weeks. At day1, 2, 3, 4, 5, 6, 7, 10, 14, 21 and 28, the plate was read by a fluorescent plate reader at 610 nm for Ru(Ph₂phen₃)Cl₂ (470 nm excitation) and 576 nm for rhodamine-B (excitation at 543 nm). Standard oxygen concentration-fluorescent intensity curve was made by reading fluorescent intensity of DPBS solution under 1, 5, 15, and 21% oxygen. Oxygen concentration of each time point was converted by the standard curve.

4.2.6 Fluorescent intensity measurement and microsphere imaging during oxygen releasing process

50mg microspheres were mixed with 1mL 2% (w/v) sodium alginate at a concentration of 50mg/mL. Alginate was chosen for the transparent property which was suite for fluorescent detection. Catalase was then added to the mixture to a final concentration of 1mg/mL. 0.2mL sodium alginate, microspheres and catalase mixture was transferred into each well of 96-well black plate. Then 30μL 400mM CaCl₂ solution was added to each well. The plate was placed steady at room temperature for a while to allow alginate gelation in the well. After the alginate/microspheres gel disk was formed in the well, 200μL DPBS was added as release medium. The release was conducted in a hypoxia incubator (1% O₂,
5% CO₂, 37°C) for 28 days. The plate was read by a fluorescent reader at day 1, 2, 3, 4, 5, 6, 7, 10, 14, 21 and 28. The excitation and emission wavelengths were set as 560nm and 655nm. Release medium was replaced with fresh DPBS every time before the plate was sent to read.

The oxygen releasing microspheres were fixed on the surface of glass slide for fluorescent imaging. PDMS was mixed with curing agent and thin film of the mixture was coated on the glass slide. Oxygen release microspheres were then quickly fabricated on the surface of PDMS pre-coated glass slide. After the PDMS film was crosslinked under 40°C in dark for 1 hour, the glass slide was incubated in DPBS in a hypoxia incubator (1% O₂, 5% CO₂, 37°C) for 28 days. The glass slide was taken out for fluorescent imaging at Day 1, 2, 3, 4, 5, 6, 7, 10, 14, 21 and 28. The microspheres on the slide were observed and images were taken by Olympus spectral FV1000 confocal microscope.

4.2.7 Toxicity test of the HYP/PVP/H₂O complex

Mesenchymal stem cells were seeded in 96 well plate with 200μL culture media and 200000 cells in each well. The plate was incubated in 5% carbon dioxide and 37°C incubator for 24 hours and then the culture media was replaced. HYP/PVP/H₂O complexes (50μL) with three different HYP amount were then added to each well with 200μL culture media and the plate was incubated in the same incubator for additional 48 hours. PVP/H₂O complex was used as control. MTT solution with a concentration of 5mg/mL in DPBS was prepared and 20 μL of solution was added to each well. Then the plate was incubated for another 4 hours. Then the media was removed carefully before 200μL DMSO was added
to each well. The plate was gently shacked until the precipitate fully dissolved. The plate was read by a plate reader at 560nm and subtract background at 670nm.

4.2.8 Mesenchymal stem cell (MSC) culture

Mouse bone marrow-derived mesenchymal stem cells (MSCs) were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) with 10% fetal bovine serum (FBS) and 1% penicillin at 37°C with 5% CO₂ and 21% O₂. Medium was changed every three days. Cells were passaged when reached 90% confluences. Cells at passages 14 - 20 were used in this experiment. Our previous works demonstrated that bmMSCs within these passages remained multipotency.

4.2.9 Encapsulate MSCs and imagable oxygen releasing microspheres into the hydrogel

The hydrogel solution was prepared by dissolving hydrogel polymer in DPBS at a concentration of 20wt% with stirring on ice. The hydrogel solution was sterilized under UV for around 30 minutes before use. Imagable oxygen releasing microspheres and catalase were added into the hydrogel solution at a concentration of 50mg/mL and 1mg/mL, respectively. MSCs were detached from the cell culture plate by Trypsin-EDTA and suspended in small amount of DPBS and added into the hydrogel solution to a final concentration of 8 million/mL. A 1mL pre-cooled syringe was used to pipette the mixture at least 20 times to make them thoroughly mixed. All the procedures above were operated on ice.
4.2.10 In vitro MSCs culture in imagable oxygen releasing system under ischemic conditions

0.2mL mixture which was made above was transferred into a 1.5mL microcentrifuge tube and the gelation was performed in 37°C water bath for 30 minutes. After the gelation, the supernatant was replaced with 200μL culture medium without FBS. The tubes which were left opened were incubated in hypoxia incubator (1% O₂, 5%CO₂, 37°C) for 7 days and the culture medium was replaced every three days. After 1, 3 and 7 days of culture, the gels were washed and then digested by 10mg/mL papain solution at 50°C for one day. Cell survival was quantified by dsDNA content (for live cells) which was measured by a Quant-iT™ PicoGreen dsDNA Assay Kit, following the provided protocol.

Gene expressions for paracrine factors were characterized by real-time RT-PCR. RNA was isolated from the gels using TRIzol following the protocol provided by Sigma. The quality of RNA was measured by NanoDrop and then used for cDNA synthesis with a High Capacity cDNA Reverse Transcription kit. Primers of forward and reverse pairs of the platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and insulin-like growth factor 1 (IGF1) were selected for gene expression study. The sequences, melting temperature, and expected product sizes are listed in Table 2.1. Real-time RT-PCR was performed three times for each sample with Maxima SYBR Green/Fluorescein master mix on an Applied Biosystems 7900 system. β-actin was used as the housekeeping gene. Fold increase was calculated using a standard ΔΔCt method.
4.2.11 Subcutaneous injection of imagable oxygen releasing microspheres with hydrogel

10% (w/v) APLA hydrogel solution was prepared and then placed under UV exposure to sterilize for 30 minutes. 50mg microspheres were added to 1mL hydrogel solutions and mixed by pipetting with a 1mL pre-cooled syringe. 200μL mixture was subcutaneously injected on mouse through 26-gauge needle. One injection site was made on each mouse. Fluorescent images of subcutaneously injected mice were taken at Day0, 3, 7, 14 by IVIS Lumina II fluorescent imaging system with excitation of 535nm and emission filter ranging from 575nm to 650nm.

After 21 days, the tissues at the injection sites of mice were collected and fixed with 4% paraformaldehyde for 24 hours. The fixed tissues were processed with paraffin embedding, sectioning and H&E staining following the standard immunohistochemistry method.

4.2.12 Statistical methods

Data are expressed as mean ± standard deviation. Statistical comparisons were performed with a One-way ANOVA by JMP. Significant difference is defined as p < 0.05.
4.3 Results

4.3.1 Polymer synthesis

The hydrogel polymer was synthesized by copolymerization of NIPAAm, APLA and HEMA using free radical polymerization method at a feed ratio of 86/4/10. The synthesis process and the structure of the synthesized polymer was confirmed by $^1$H-NMR spectrum as showed in Chapter 2. From the spectrum, the molecular ratio of each component in the polymer NIPAAm/APLA/HEMA was determined as 86/4.2/9.8 which was consistent with the feed ratio.

The hydrogel solution was in liquid form and flowable at 4°C. It was able to inject through a 26-gauge needle which was usually used in surgery of tissue for cells injection (Figure 4.2). The hydrogel solution possessed a thermal transition temperature of 26.5 ± 0.2°C. The hydrogel was able to solidify under 37°C in 7 second. The short gelation time helped ensure the gel retain in the heart after injection. After equilibrium the gel solution in 37°C, the soft gel had a water content of 48.7 ± 4.17 %. It showed highly flexibility with a Young’s modulus of 35 ± 5 kPa. When the gel was incubated at 37°C in DPBS, it degraded slowly and had a weight remaining of 93.3% after 8 weeks. The degradation product was soluble at 37°C because the thermal transition temperature of degraded hydrogel was raised to 42°C, higher than 37°C. The degradation product was proved to be non-toxic to cells.
4.3.2 HYP/PVP/H₂O₂ complex spectral properties characterization

The fluorescent emission spectrum of HYP/PVP/H₂O₂ complex was obtained by scanning emission intensity from 570nm to 800nm with excitation of 555nm following the reported parameters (Figure 4.3). From the acquired spectrum, the emission intensity started to increase at around 625nm and reach the highest peak at around 650nm. The high emission continuous and slowly dropped after 700nm. The spectrum of complex solution showed there was a shift of emission comparing to the HYP/PVP/H₂O complex. And from the result, wavelength number between 650 to 700nm could be used for emission detection.

4.3.3 HYP oxygen releasing microspheres characterization

The imagable oxygen releasing microspheres were fabricated by electrospray method. SEM images of the microspheres were taken to illustrate the microstructure of the fabricated microspheres as showed in Chapter 3. The diameter of the microspheres was around 5μm. The core shell structure of the microspheres could be characterized by confocal microscope. The confocal image (Figure 4.4) demonstrated the microsphere was core-shell structured with PLGA (indicated in red color) as shell and HYP/PVP/H₂O₂ as core (indicated in yellow color).

4.3.4 HYP ORM oxygen releasing kinetics

To investigate whether oxygen release from the microspheres could perform normally before and after HYP was added to the core complex, oxygen releasing kinetics of 4 kinds
of microspheres were conducted in hypoxia incubator for 28 days. The release of oxygen came from \( \text{H}_2\text{O}_2 \) catalyzed by catalase when HYP/\( \text{H}_2\text{O}_2/\text{PVP} \) complex was slowly and continuously released from the microspheres when the shell PLGA degraded. For the reason that \( \text{H}_2\text{O}_2 \) was in complex form conjugated with PVP, \( \text{H}_2\text{O}_2 \) was stabilized and thus the releasing of \( \text{H}_2\text{O}_2 \) could last for a relatively long period of time. The oxygen release kinetics was measured by converting fluorescent intensity of oxygen-sensitive luminophore referring to oxygen-insensitive fluorophore to oxygen concentration with a standard curve. The oxygen release curve showed microspheres with three different HYP/\( \text{H}_2\text{O}_2/\text{PVP} \) ratio can sustainably release oxygen for 4 weeks (Figure 4.5). The normal oxygen releasing microspheres were used as control. At the beginning, the oxygen released slowly among all microspheres conditions until Day 3. There was a burst release that appeared in all conditions at day 4 and the oxygen level quickly reached the highest point at Day 5. Even though the oxygen level decreased a little, sustained oxygen release was observed in the following days until Day 28. In first 3 days, there was no big release difference among the 4 type microspheres. During this time, the complex in the shell came out from the microspheres which resulted a small amount of oxygen release of around 7% (21% oxygen in normal air). At Day 4, burst release of all conditions was observed. The oxygen level of control and 10HYP were around 18%, 15HYP was 24% and 5HYP was 14.9%. Oxygen level of 5HYP oxygen releasing microspheres increased slowly to 18.2% at Day 5 and then went down but kept releasing oxygen at around 10% until day 28. Oxygen level of 15HYP oxygen releasing microspheres steadily increased to 24.5% on Day 5 and slowly dropped to the range between 10 - 20% in the following 23 days. Among the three
imagable oxygen releasing microspheres, 10HYP oxygen releasing microspheres performed very similar oxygen release kinetics to the control. The oxygen level reached the highest 24.6% at Day 5 and then decreased to 11.7% at Day 28. The oxygen release kinetics of HYP imagable oxygen releasing microspheres demonstrated that the newly developed microspheres could release oxygen normally as before. The release amount of oxygen was related to the amount of HYP which was added to the complex. Among the three different HYP conditions, more HYP added to the complex, more oxygen was released from the microspheres.

4.3.5 HYP based imagable ORM in vitro study

The applicable of HYP based fluorescent imagable oxygen releasing microspheres was studied by fluorescent plate reader and FV2000 spectra confocal microscope in vitro. Fluorescent intensity of three kinds microspheres with different HYP amount was read at pre-determined time point in a 2-week time period while the fluorescent images were taken in a 4-week time period (Figure 4.6). The fluorescent intensity of the microspheres decreased with increasing time due to the release of fluorescent agent HYP to the releasing medium. In the beginning, slight change of the fluorescent intensity was observed for three conditions and there was an obvious intensity drop on Day 3. Then the intensity slowly decreased with time. During the releasing period, the intensity of microspheres was tightly related to the amount of HYP in the microspheres. 15HYP microspheres showed high fluorescent intensity from the beginning of the release until the end. This result could also be confirmed by the confocal images of microspheres (Figure 4.7). The red color of the
microspheres faded with time and more intense red color was observed on 15HYP microspheres than the other two kinds of microspheres. The results also indicated that the developed imagable oxygen releasing microspheres which incubated in releasing medium under ischemic conditions could be easily imaging under confocal microscope for at least 21 days *in vitro*.

4.3.6 *MSC survival in HYP ORM encapsulated hydrogel system in vitro*

Before MSCs culture in the imagable oxygen releasing system, whether the released complex would affect cell growth was tested by MTT assay. In Figure 4.8, the cell viability of groups with adding the released complex of three different HYP content were similar to the control. The result indicated that the HYP/PVP/H₂O complex was not toxic to the cells in normal culture conditions.

MSC survival in the imagable oxygen releasing system was characterized by measuring the dsDNA content. MSC culture in normal oxygen releasing system was treated as control. The dsDNA results (Figure 4.9) demonstrated that MSCs culture in normal and imagable oxygen releasing system could survive in ischemic conditions. The cell could even proliferate and the cell numbers doubled after 7 days culture. In normal ORM group, the number of MSCs did not significantly change on Day 3. And the cell number increased to 225.7% on Day 7. For three HYP microspheres groups, number of MSCs decreased slightly on Day 3. However, the MSCs proliferated afterwards and on Day 7, the cell number raised to more than 200% than on the day1 and cell number almost reached 300%
on 15HYP group which meant the HYP based imagable oxygen releasing system was able promote cell survival. Live cell images were consistent with the dsDNA data (Figure 4.10).

Real-time RT-PCR was used to examine the paracrine effect at mRNA level. From the gene expression result, MSCs cultured in the imagable and normal oxygen releasing system all exhibited expression of four paracrine factors. PDGF, HGF, IGF1 and VEGF compared to the non-oxygen releasing MSCs (Figure 4.11). Among them, PDGF and IGF1 increased significantly. These indicated that the survived CDCs were able to secrete paracrine factors which in turn enhanced cell survival and proliferation. Interestingly, MSCs in imagable oxygen releasing system showed higher gene expression of the VEGF than the normal oxygen releasing groups.

4.3.7 HYP based Imagable oxygen releasing system in vivo

The imagable oxygen releasing system was injected subcutaneously and the oxygen releasing microspheres could be visualized in fluorescent imaging machine for 14 days. Series of images were taken (Figure 4.12) at different time points and the fluorescent changing in vivo were investigated. The intensity of the imaged area of oxygen releasing system was related to the amount of HYP in the microspheres. 15HYP showed highest fluorescent intensity. The intensity of the three groups drops with time until no fluorescent color could be observed. Among them, 15HYP oxygen releasing microspheres showed highest fluorescent intensity with long time in vivo during the whole experiment period and could still be visualized after 14 days. On Day 3, the intensity of all three groups decreased a little. On Day 7, no fluorescent area could be found on the mouse back. On Day14, the
color of 10HYP faded away and only 15HYP group showed obvious fluorescent area on the mouse back. As showed in Figure 4.13, H&E images of the subcutaneous tissues showed that the imagable oxygen releasing system was not toxic to tissues in vivo and won’t cause tissue inflammation or abnormal functioning.

4.4 Discussion

Currently many oxygen releasing systems were developed for tissue regeneration applications to address the low oxygen environment problem. Our previously fabricated core-shell structured oxygen releasing microspheres could release oxygen for relatively long time to support stem cell survival under ischemic conditions. However, the system applied site and the releasing process were not visible. This study demonstrated that HYP based imagable oxygen releasing microspheres were fabricated and could be easily imaged both in vitro and in vivo while generating oxygen at the same time as well.

The oxygen releasing kinetics showed different oxygen releasing behavior of the three type microspheres with different HYP. The highest HYP microspheres (15HYP) released the considerably highest level of oxygen (~24%) on Day 4 while the remaining two microspheres released less oxygen. The release difference among the different conditions might be a result of conjugation competition between HYP to PVP and H$_2$O$_2$ to PVP. In the mixture solution for the core, both HYP and H$_2$O$_2$ molecules could conjugate with PVP to form a complex. More HYP appeared in the mixture, less H$_2$O$_2$ could conjugate with PVP. Thus, more free H$_2$O$_2$ was released and quickly converted to oxygen by catalase. In other words, less H$_2$O$_2$ was trapped in the complex. This could be demonstrated from the
releasing behaviors among the three kinds of microspheres after Day 6. Even though 10HYP and 15HYP microspheres released more oxygen before Day 6, the oxygen release started to drop afterwards. However, the 5HYP microspheres kept releasing oxygen in a relatively steady manner until Day 28.

Burst of oxygen release started to happen after Day 3. At this time, the release of the complex trapped in the inner shell was almost over and the complex from the core began to release out of the shell. Interestingly, the releasing kinetics coordinated with the fluorescent intensity changing during the releasing process. The burst releasing oxygen resulted from the complex releasing from the core. And the quick releasing of complex from the core caused the HYP amount reduction in the microspheres which led to a significant fluorescent intensity decrease. The intensity continued to decrease but the rate slowed down afterwards while the oxygen release of the microspheres tended to be low but sustainable.

To investigate the oxygen supply function of the imagable oxygen releasing microspheres, cell survival study was conducted in vitro by culturing MSCs in the hydrogel together with the imagable oxygen releasing microspheres under ischemic conditions. The oxygen release was realized when the H₂O₂ in the released complex was converted by catalase to oxygen and water. After that the remaining complex HYP/PVP/H₂O was left in the medium and they could contact with the cell. Whether the HYP/PVP/H₂O complex could affect cell viability was a big concern. By performing MTT assay to test the toxicity of HYP/PVP/H₂O to cells, we found that the complex did not show negative effect on cell growth. dsDNA result demonstrated that the newly developed system was still able to
promote cell survival under ischemic conditions comparing with the old oxygen releasing system. The only difference appeared in the cell numbers at Day 3. For the control, almost no cell death was observed at Day 3. But among HYP oxygen releasing microspheres groups, around 10% cells death was observed at Day 3. The performance of cell survival was closely related to the oxygen supply to the cell cultured in hypoxia. From the releasing curves of the microspheres, it could be see that the released amount of oxygen was very low (3% lower than the control) in the first three days. Therefore, this situation happened due to the compromised oxygen releasing conditions of the microspheres. The low amount of oxygen could not support cell survival in the harsh conditions and partial cells died as a result. However, cell number elevation was observed on Day 7 from dsDNA data and live cell images because after Day 3 there came an oxygen release burst and the release kept at a relatively high level for a long time. With sufficient oxygen in the environment, cells were able to survive and proliferate.

The gene expression of the survived cells in oxygen releasing system showed higher expression of growth factor gene markers under ischemic culture conditions after 7 days cell culture. Significant fold increase of IGF1 indicated that the proliferation ability of the survived cells was stimulated and upregulated by the released oxygen and ischemic environment. In addition, the survived cells had high expression of PDFGA as well as elevated expression of HGF and VEGF. The stimulated paracrine effect resulted in the growth factors secretion from the survived cells. The growth factor in turn helped promote cell survival, proliferation and differentiation as well as angiogenesis in the tissue.
In vivo injection of the imagable oxygen releasing system subcutaneously could be detected. HYP complex released from the microspheres could also be visualized for some time as the activity of HYP was stabilized with BSA as reported [216]. In vivo imaging was consistent with the in vitro results. The image of 5HYP and 10HYP microspheres fade away in short time as the fluorescent intensity decreased significantly to beneath the lower level of detection. Only 15HYP group could guarantee solid detection for 2 weeks. To ensure long term visualization of the microspheres to accompany the long term oxygen release for 4 weeks, larger amount of HYP should added to the system. The very encouraging thing was the imagable oxygen releasing system showed non-toxicity to the host tissue as showed in H&E images of which the tissues were identified normal without inflammation.

4.5 Conclusion

In this work, an imagable oxygen releasing system was successfully developed. The oxygen releasing function was based on previously fabricated core-shell structured microspheres and the microspheres was visualized by adding fluorescent agent HYP into the releasing complex PVP/H$_2$O$_2$ to form a new complex. From in vitro and in vivo study, the imagable oxygen releasing system was able to release oxygen at certain satisfactory levels for at least 14 days to promote cell survival under ischemic conditions, and at the same time be fluorescently imaged. And the fluorescent intensity correlated with the releasing time. This imagable system provided a promising and beneficial way in clinical trials in helping monitoring the conditions of oxygen releasing microspheres in vivo by
simply visualizing the injected oxygen releasing system in the machine without any invasion.
Figure 4.1 Scheme of co-axial electrospray technique to fabricate HYP based imagable oxygen releasing microspheres.
Figure 4.2  (A) Hydrogel solution was flowable at 4°C; (B) Hydrogel solution forms gel at 37°C; (C) and (D) solid hydrogel was flexible.
Figure 4.3  Structure of HYP/PVP complex and the emission spectra of HYP/PVP complex (Excitation: 555nm).
Figure 4.4 Confocal images of core-shell structure of imagable oxygen releasing microspheres. HYP/PVP H$_2$O$_2$ complex and PLGA are core and shell, respectively. For imaging purpose, Rhodamine-B were added to the shell. The shell structure was indicated in red color while the core was indicated in yellow color. Scar bar: 5 μm.
Figure 4.5  Oxygen releasing kinetics of imagable oxygen releasing microspheres with 3 different HYP amount (5HYP, 10HYP, and 15HYP). PVP/H₂O₂ microspheres was used as control.
Figure 4.6 Fluorescent intensity of 3 type imageable oxygen releasing microspheres incubated in DPBS at 37°C for 14 days.
Figure 4.7 Fluorescent images of 3 type imagable oxygen releasing microspheres incubated in DPBS at 37°C for 14 days. Scale bar: 5μm.
Figure 4.8  Toxicity test of the released complex HYP/PVP/H$_2$O. PVP/H$_2$O was used as control.
Figure 4.9  dsDNA content of MSC cultured in hydrogel with 5HYP, 10HYP, and 15HYP ORM under ischemic conditions for 7 days.
Figure 4.10 Live cell images of MSC cultured in hydrogel with 5HYP, 10HYP, and 15HYP ORM under ischemic conditions at Day1, 3, and 7. Scale bar: 50μm.
Figure 4.11 Paracrine effect (PDGFB, HGF, IGF1, and VEGFA) of imagable oxygen releasing microspheres with 3 different HYP amount (5HYP, 10HYP, and 15HYP). Microspheres with PVP/H$_2$O$_2$ as core were used as control.
Figure 4.12  *In vivo* fluorescent images of subcutaneous injection of imagable oxygen releasing microspheres with 3 different HYP amount (5HYP, 10HYP, and 15HYP) for 14 days.
Figure 4.13  H&E images of tissue sections after subcutaneous injection of imagable oxygen releasing microspheres with 3 different HYP amount (5HYP, 10HYP, and 15HYP) after 21 days.
CHAPTER 5: Injectable thermosensitive hydrogel based hypoxia-sensitive oxygen releasing system to augment mesenchymal stem cells survival under ischemic conditions

5.1 Introduction

Ischemic disease is caused by local loss of blood supply to the bodily organ, tissue or part due to mechanical obstruction of the blood vessels, mainly arterial narrowing or disruption [217]. Brain ischemia, myocardial ischemia and limb ischemia are three major ischemic diseases which affect people worldwide and cause large death rate among the patients [189-192]. Restricted blood supply to the affected tissue areas causes ischemic environment (low oxygen and low nutrients) which leads to quick cell death due to the activation of apoptosis pathway and then followed by tissue necrosis. The functions of organs are lost as a result of tissue damage.

In order to regenerate the damaged tissues caused by ischemia, stem cell therapy is regarded as promising therapeutic approach [218-220]. In the therapy, stem cells are injected to the target tissue area. The transplanted stem cells are expected to attach, proliferate and differentiate in the injection site and surrounding area to regenerate the damaged tissues and recover the lost functions. However, the extremely low cell survival during transplantation and under ischemic conditions after injection are considered as big challenges to the treatment [68, 98, 221]. To make a proper cell transportation vehicle,
biodegradable thermosensitive hydrogel developed in our lab could be used. The previous study demonstrated that the hydrogel could encapsulate cells as well as growth factors and oxygen releasing microspheres [54, 90, 130, 131]. It showed biocompatibility to the cells and offered a proper microenvironment for cells attachment and growth. Mesenchymal stem cell (MSC) have great self-renewal ability while maintaining its multipotency. MSCs were reported to differentiate into multiple cell types in vitro and in vivo, including myocytes and neurons [198-200]. Even though the differentiation of MSCs varies among individuals, it has been also widely investigated that the differentiation could be chemically or mechanically induced. Several studies in our lab demonstrated that MSCs differentiation were related and could be controlled by the mechanical properties of the cell carrier matrix – scaffolds or hydrogels [28, 131-133, 178, 222]. Thus the MSC/hydrogel deliver system could be applied into cardiac, brain or skeletal muscles tissue regeneration.

A basic oxygen releasing system was fabricated and functioning properly to support stem cell survival under ischemic conditions in Chapter 3. The oxygen releasing microsphere used PLGA as shell material [178]. The content releasing kinetics depended on the degradation rate of selected PLGA. This meant that the releasing behavior was fixed once a shell material was chosen. However, in real tissue regeneration circumstances, relatively large amount of oxygen was required when the oxygen content in the damaged tissue environment was extremely low [194-197]. Oxygen demand in different tissues varied and once the oxygen level recovered or oxygen content in the tissue area was not that low, less oxygen was needed [223]. The abundant oxygen might induce reactive oxygen species (ROS) which stimulated cell apoptosis [54, 103, 224]. The currently
reported oxygen releasing systems, even though they were controllable due to the properties of materials and oxygen generators, were unable to adjust oxygen release according to the environment [85-89, 170, 201, 225, 226]. In order to change the oxygen releasing behavior of the microspheres to fit the practical requirement, shell material needs to be changed accordingly. As we know, the degradation of PLGA follows bulk degradation mechanism [48, 90, 227]. More hydrophilicity and water content in the material result in faster degradation of the polymer. Based on the above scenario, we expects that by using a hypoxia-sensitive material of which the hydrophilic property improved under hypoxic condition, oxygen releasing behavior of the microsphere could be controlled and altered depending on the oxygen content in the environment.

It was reported that 2-nitroimidazole (NI), a hydrophobic compound that can be converted to hydrophilic 2-aminoimidazoles through bioreduction under hypoxic conditions [228, 229]. In the study, this hypoxia-sensitive component was used to modify hyaluronic acid to realize a controllable glucose oxidase and insulin. It was demonstrated that the modified polymer would respond to hypoxia, turn from hydrophobic to hydrophilic.

We hypothesized that by modifying the degradable shell materials with NI, hypoxia-sensitive oxygen releasing microspheres could be developed. The shell material of the oxygen releasing microspheres would turn to be more hydrophilic under hypoxic conditions. Thus the oxygen releasing behavior should change and a faster and more amount of oxygen release should be performed accordingly.

The newly developed polymer as the shell material was based on APLA which was the degradable component and NAS which could be functionalized via simple organic
conjugation reaction [138]. Modification of catalase on ORM was obtained via a one-step conjugation reaction between amine group on catalase and NHS groups on the shell polymer material.

Here we reported a novel hypoxia-sensitive oxygen releasing system based on biodegradable thermosensitive hydrogel loaded with hypoxia-sensitive oxygen releasing microspheres. APLA hydrogel was again in use to serve as a carrier the system. This injectable hydrogel was demonstrated previously to be a proper transplantation carrier with good biocompatibility to deliver cells for tissue regeneration and could be easily operated both in vitro and in vivo. Meanwhile, the oxygen permeability of the hydrogel allowed perfect oxygen dispersion in the hydrogel which made it an ideal vehicle for oxygen releasing microspheres encapsulation. In the system, instead of using the basic PLGA microspheres, a hypoxia-responsive organic compound NI was introduced to functionalize the newly developed degradable shell polymer material for oxygen releasing microsphere to fulfill the hypoxia-sensitive property. In this work, we demonstrated that the core-shell structured hypoxia-sensitive oxygen releasing microspheres were fabricated and the hydrophilicity change of the shell material of the microspheres under hypoxia conditions were expected to result in higher degradation rate which led to fast and high oxygen release response from the oxygen releasing microspheres. Further catalase modification on the microsphere surface eliminated the catalase adding procedure and therefore should make the oxygen releasing system more convenient in practical application. In addition, the newly developed oxygen releasing system could be used to enhance cell survival under
ischemic cell culture. The approach provided an efficient and novel therapeutic method for stem cell therapy to treat ischemia induced tissue damage.

5.2 Materials and methods

5.2.1 Materials

N-isopropylacrylamide (NIPAAM, TCI) was recrystallized with hexane for three times before use. 2-Hydroxyethyl methacrylate (HEMA, Alfa Aesar), DL-α-hydroxy-β,β-dimethyl-γ-butyrolactone, trimethylamine, N-Hydroxysuccinimide, acryloyl chloride, hydrogen peroxide (30% aqueous solution), bovine liver catalase (2000-5000 units/mg), poly (N-vinylpyrrolidone) (PVP) with molecular weight 40-90 kDa, Ru(Ph$_2$phen)$_3$Cl$_2$, and rhodamine-B, Boc$_2$O, nitroimidazole and 2-bromoethylamin hydrobromide were from Sigma and used as received.

5.2.2 Synthesis 2-(2-Nitroimidazolyl) ethanamine (NI)

First tert-butyl (2-bromoethyl)carbamate was synthesized by reaction Boc$_2$O and 2-bromoethylamin hydrobromide. A 25mL flask was charged with Boc$_2$O (533mg, 2.44mmol) and 2-bromoethylamin hydrobromide (556mg, 2.71mmol) at 0°C followed by the dropwise addition of TEA (508µL, 3.66mmol) over 10min. The reaction was allowed to warm to room temperature and continued to react for an additional of 18h. The colorless reaction mixture was diluted with CH$_2$Cl$_2$ (100mL) and washed with sat aq NH$_4$Cl (20mL), sat aq NaHCO$_3$ (20mL) and brine (20mL). The combined organic layers were dried over
anhydrous Na₂SO₄, filtered and concentrated under reduced pressure to obtain pure tert-butyl (2-bromoethyl)carbamate which was used as a linker to conjugate with a nitroimidazole group on one side and a chelating group on the other (Figure 5.1) [229]. Compound in the next step was synthesized with tert-butyl (2-bromoethyl)carbamate and nitroimidazole by using K₂CO₃ as a base in dimethylformamide (DMF). The reaction was conducted at 80°C for 4h and then continued overnight with stirring at room temperature. The product was recrystallized in EtOAc. Final compound NI was obtained when 1.25 M HCl in MeOH was used to deprotect the amine group. The reaction was proceeded at room temperature for 24h. NI was recrystallized in MeOH and vacuum dried before use. The structure of NI was confirmed by ¹H-NMR.

5.2.3 Synthesis N-Acryloxysuccinimide (NAS)

115g N-Hydroxysuccinimide (NHS) and 110g trimethylamine were dissolved in 1500mL chloroform at 0°C. Acryloyl chloride (100g) was added dropwise over a 20 minutes period to the mixture with stirring. After the mixture was stirred for an additional 20 minutes, the solution was washed with 800mL ice cold water and saturated brine, dried with anhydrous MgSO₄ and vacuum filtered. A mixture of 30mL ethyl acetate and 200mL hexane was added slowly with vigorous stirring to the filtered solution. The solution was then left to stand at 0°C for several hours to allow crystallization. The precipitated, colorless crystals were separated by filtration and washed with ice cold 100mL hexane/ethyl acetate (4/1), 100mL hexane/ethyl acetate (9/1), finally with two portions of
100mL hexane. The crystals were dried in vacuum at ambient temperature to constant weight and stored in -20°C before use. The structure was confirmed by $^1$H-NMR.

5.2.4 Synthesis polymer NIPAAm-APLA-NAS-HEMA

Monomer APLA was synthesis following the procedures described in Chapter 2. Polymer NIPAAm-APLA-NAS-HEMA (H5A25N20) with monomer molar ratio of 50/25/20/5 was polymerized via free radical polymerization method. Pre-determined amount of NIPAAm (1.93g), APLA (2.70g), NAS (1.15g), and HEMA (0.22g) were charged in a 250mL three neck round bottom flask and dissolved in 110mL dioxane. The reaction was initiated by 16mg BPO (freeze dried before use) and proceeded under nitrogen protection in 70°C oil bath overnight. To obtain the polymer, the solution was poured into cold hexane to precipitate out the polymer. The solid polymer was then purified by dissolving in THF and precipitating in anhydrous ethyl ether twice. The purified polymer was vacuum filtered and vacuum dried in oven until no weight change before use. The polymerization reaction was showed in Figure 5.2. The structure and actual molar ratio of the polymer were confirmed by $^1$H-NMR.

5.2.5 Conjugate 2-(2-Nitroimidazolyl)ethanamine (NI) on polymer NIPAAm-APLA-NAS-HEMA

NI conjugated polymer H5A25NI10 was synthesized by conjugation reaction between H5A25N20 and NI. To be specific, 1g H5A25N20 polymer and 0.09g NI were dissolved
in 20mL DMF with addition of 0.08mL TEA in a round bottom flask. The reaction was conducted under protection of nitrogen at around 60°C overnight. After the solvent DMF was eliminated by rotary evaporation, the product was then dissolved with around 5mL THF and precipitated in anhydrous ethyl ether. The precipitate polymer was obtained by centrifuge at 8000rpm and vacuum dried before use. Reaction was demonstrated in Figure 5.3. The structure and molar ratio of each component were confirmed and calculated by $^1$H-NMR.

5.2.6 Polymer properties characterization

The thermal transition temperature of the solution was measured by using differential scanning calorimetry (DSC) over a temperature range of -80-200°C, with a heating rate of 20°C /min. 10mg solid polymer was sealed in an aluminum pan and placed in the DSC machine. An empty pan was used as a reference.

Water content of H5A25NI20 and H5A25NI10 were measured in normal and hypoxic conditions. Both of the solid polymers were dissolved in DCM to obtain solutions with a concentration of 10% (w/v). 0.2mL polymer solution was added to each microcentrifuge tube. After the solvent was evaporated, 200μL DPBS was added. The tubes were incubated in 1% and 21% oxygen conditions at 37°C for 24 hours and the DPBS in the tube was discarded. The wet weight of the polymer was measured as $w_1$, while the lyophilized hydrogel weight was measured as $w_2$. The water content was calculated as:

$$\text{Water content (\%)} = \frac{(w_1 - w_2)}{w_2} \times 100\%$$
Degradation of microsphere shell materials in DPBS in normal and hypoxic conditions were conducted for 8 weeks. 200uL of polymer solution were added to a 1.5mL microcentrifuge tubes and the tubes were placed in a 37°C water bath. The tubes with a hole on caps were incubated in normal (21%O₂, 5%CO₂, 37°C) and hypoxic (1%O₂, 5%CO₂, 37°C) incubator. The tubes were taken at each time point. After the DPBS was discarded, polymer was freeze dried and the dry weight of polymer was measured as w₃. The weight of polymer before degradation (week 0) was w₄. The weight remaining was calculated as:

\[ \text{Weight remaining} \% = \frac{w_3}{w_4} \times 100\% \]

5.2.7 Hypoxia sensitive oxygen releasing microspheres fabrication

To fabricate the hypoxia sensitive oxygen releasing microspheres, H5A25NI20 and H5A25NI10 polymers were used as shell materials and PVP/H₂O₂(1/4.5) complex was used as core. The shell materials polymer solution was prepared by dissolving the polymer into DCM at a concentration of 5wt%. Microspheres were fabricated by co-axial electrospinning device with established electrospray technique which was introduced in Chapter 3 in detail. Microspheres which were made with non NI conjugated polymer as shell were treated as control.
5.2.8 Catalase conjugated hypoxia sensitive oxygen releasing microspheres fabrication and catalase conjugation density measurement

To fabricate the catalase conjugated hypoxia sensitive oxygen releasing microspheres, H5A25NI20 and H5A25N20 polymers mixture were used as shell materials and PVP/H$_2$O$_2$(1/4.5) complex was used as core. The shell materials polymer solution was prepared by dissolving the polymer into DCM at a concentration of 5wt% with H5A25NI20/H5A25N20 molar ratio of 90/10, 70/30, 50/50 and 0/100. Microspheres were fabricated by co-axial electrospinning device with established electrospray technique which was introduced in chapter 3 in detail. Microspheres which were made with H5A25NI20/H5A25N20 molar ratio of 0/100 as shell material were treated as control.

To conjugate the catalase on the surface of the microspheres and determine the conjugation density, catalase was first labelled with FITC following the method described in Chapter 3. Then the 10mg/mL catalase-FITC solution was dialysis against pH 8.0 0.1M sodium phosphate buffer at 4°C for 24 hours. The dialysis buffer was replaced and the dialysis was proceeded for another 24 hours. The 50mg of dry powdered microspheres was weighed and added to the 6mL 5mg/mL catalase-FITC solutions. The mixture was stirred at 4°C for 4 hours to allow conjugation of catalase to the NHS groups on the surface of microspheres. The catalase conjugated hypoxia sensitive oxygen release microspheres were obtained by centrifuge at 10000rpm and then were freeze dried before use. Fluorescent intensities of the catalase-FITC solutions before and after conjugation were measured by fluorescent reader. The amount of catalase could be calculated by converting the intensity from the standard curve of the fluorescent intensity vs concentration. The
conjugation density of catalase on the microspheres could be calculated with the formula below:

$$\text{Conjugation density} = \frac{(m_1-m_2)}{m_0},$$

$m_0$ was the amount of microspheres added to the catalase solutions, $m_1$ and $m_2$ were weight of catalase in the solution before and after conjugation process.

### 5.2.9 Oxygen releasing kinetics

The hypoxia sensitive oxygen release microspheres and catalase conjugated hypoxia sensitive oxygen release microspheres with different NI amount and different NI/CAT ratio were fabricated and carefully stored. Sustained oxygen releasing was conducted for 4 weeks by detecting the intensity of oxygen sensitive fluorescent dye. To achieve this, an oxygen-sensitive PDMS membrane was made with luminophore Ru(Ph₃phen)Cl₂ and oxygen-insensitive fluorophore rhodamine-B as described in chapter 3. The membrane was cut to disk shape of 6mm diameter and placed into wells of a 96-well black plate. After 200μL DPBS and 1mg/mL bovine catalase (if no catalase was conjugated on the microspheres) were added to each well, the plate was incubated in 1% oxygen incubator overnight to balance the oxygen content. Then 10mg microspheres was quickly added to each well and the plate was sealed with transparent film. The oxygen release process was performed in 1% oxygen and 37°C incubator for 4 weeks. At Day1, 2, 3, 4, 5, 6, 7, 10, 14, 21 and 28, the plate was read by a fluorescent plate reader at 610 nm for Ru(Ph₃phen)Cl₂ (470 nm excitation) and 576 nm for rhodamine-B (excitation at 543 nm). Standard oxygen concentration-fluorescent intensity curve was made by reading fluorescent intensity of
DPBS solution under 1, 5, 15, and 21% oxygen. Oxygen condition of each time point was calculated by the standard curve.

5.2.10 Mesenchymal stem cell culture

Mouse bone marrow-derived mesenchymal stem cells (MSCs) were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) with 10% fetal bovine serum (FBS) and 1% penicillin at 37°C with 5% CO₂ and 21% O₂. Medium was changed every three days. Cells were passaged when reached 90% confluences. Cells at passages 14 - 20 were used in this experiment. Our previous works demonstrated that bmMSCs within these passages remained multipotency.

5.2.11 Encapsulate hypoxia-sensitive oxygen releasing microspheres and MSCs into APLA hydrogel

To investigate the cell survival in hypoxia-sensitive oxygen releasing microspheres encapsulated hydrogel system, previously developed APLA hydrogel (NIPAAm/APLA/HEMA = 86/4/10) was introduced to use. The APLA hydrogel solution was prepared by dissolving hydrogel polymer in DPBS at a concentration of 20wt% with stirring on ice. The hydrogel solution was sterilized under UV for around 30 minutes before use. Hypoxia-sensitive oxygen releasing microspheres were added into the hydrogel solution at a concentration of 50mg/mL. For the microspheres without catalase conjugation, catalase was added at a concentration of 1mg/mL. MSCs were detached from the cell
culture plate by trypsin and suspended in small amount of DPBS and added into the hydrogel solution to a final concentration of 8 million/mL. A 1mL pre-cooled syringe was used to pipette the mixture at least 20 times to make them thoroughly mixed. All the procedures above were operated on ice.

5.2.12 In vitro MSCs culture in hypoxia-sensitive oxygen releasing microspheres encapsulated hydrogel system

0.2mL mixture which was made above was transferred into a 1.5mL microcentrifuge tube and the gelation was performed in 37°C water bath for 30 minutes. After the gelation, the supernatant was replaced with 200μL culture medium without FBS. The tubes which were left opened were incubated in hypoxia incubator (1% O₂, 5%CO₂, 37°C) for 7 days and the culture medium was replaced every three days. After 1, 3 and 7 days of culture, the gels were washed and then digested by papain solution with overnight incubation at 50°C. Cell survival was quantified by dsDNA content (for live cells) which was measured by a Quant-iT™ PicoGreen dsDNA Assay Kit, following the provided protocol.

Gene expressions for paracrine factors were characterized by real-time RT-PCR. RNA was isolated from the gels using TRIzol following the protocol provided by Sigma. The quality of RNA was measured by NanoDrop and then used for cDNA synthesis with a High Capacity cDNA Reverse Transcription kit. Primers of forward and reverse pairs of the platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and insulin-like growth factor 1 (IGF1) were selected for gene expression study. The sequences, melting temperature, and expected product sizes are
listed in Table 2.1. Real-time RT-PCR was performed three times for each sample with Maxima SYBR Green/Fluorescein master mix on an Applied Biosystems 7900 system. \(\beta\)-actin was used as the housekeeping gene. Fold increase was calculated using a standard \(\Delta\Delta C_{t}\) method.

5.2.13 Statistical methods

Data are expressed as mean ± standard deviation. Statistical comparisons were performed by ANOVA using JMP. Significant difference is defined as \(p < 0.05\).

5.3 Results

5.3.1. Hypoxia-sensitive degradable polymer synthesis and properties

The hypoxia-sensitive compound was synthesized following the reported method (Figure 5.1). Amine group was preserved for further conjugation reactions. The biodegradable polymer was synthesized by copolymerization of NIPAAm, APLA, NAS and HEMA using free radical polymerization method with a feed ratio of 50/25/20/5 (Figure 5.2). Hypoxia-sensitive degradable polymer was synthesized by conjugating NI-amine on the H5A25N20 polymer via simple amine-NHS reactions (Figure 5.3). The structures of the synthesized compound and polymers were confirmed by \(^1\text{H}\)-NMR spectrum (Figure 5.4, Figure 5.5, and Figure 5.6). The molecular ratio of NIPAAm/APLA/NAS/HEMA was consistent with the feed ratio. And the amount of NI conjugated on the polymer with ratio 10 and 20 was made. In Table 5.1, glass transition
temperature (Tg) of the H5A25N20 was determined to be 56°C by DSC. After NI conjugation, Tg of the hypoxia-sensitive polymer decreased to 45°C and 48°C for H5A25N20-20NI and H5A25N20-10NI. More NI conjugation made lower Tg of the polymer.

Water contact angle demonstrated the wettability of the polymer surface. Higher amount of NI conjugation on the polymer make the polymer more easily wetted due to the increasing hydrophilic of the polymer after incubation in hypoxic conditions. After incubating the hypoxia-sensitive polymer in DPBS at 37°C in both normal and hypoxia conditions, the polymer had different water content (Table 5.2). The water content of 20NI polymer was 89.64% in normal condition and 153.52% in hypoxic condition. The water content of 10NI polymer was 74.22% in normal condition and 91.86% in hypoxic condition.

Degradation properties of the hypoxia-sensitive was conduction in DPBS under both normal and hypoxic conditions at 37°C for 8 weeks. From Figure 5.7, both polymers degraded quickly in the first week and then the degradation rate slowed down. The degradation behavior of the hypoxia-sensitive polymer should relate to the amount of conjugated NI and the oxygen conditions in the environment. Compared with 20NI and 10NI polymers, the degradation curve showed 5% more weight loss of 20NI polymer under hypoxic conditions. There was a 20% weight loss of 10NI polymer under normal conditions and 25% weight loss under hypoxic conditions. Higher weight loss was observed on 20NI polymer. In normal condition, the weight remaining of 20NI polymer was 76.6%, and while in hypoxic conditions weight remaining dropped to 71.3%.
5.3.2. Fabrication hypoxia-sensitive oxygen releasing microspheres and oxygen release kinetics

The hypoxia-sensitive oxygen release microspheres were fabricated with a co-axial device using electrospray technique. By controlling the applied voltages of the electrical field and infusion rate of H₂O₂/PVP complex and hypoxia-sensitive polymer solution, the core-shell structure microspheres were fabricated from the device. The morphology of microspheres was showed in SEM image and the core-shell structure was showed by confocal images of the microspheres of which the core and shell are labeled with different fluorescent dyes (Figure 5.8). By using different NI conjugated polymer as shell materials, four kinds of microspheres were prepared to investigate different oxygen generation (Figure 5.9). Microspheres with no NI conjugation polymer H5A25N20 as shell and PLGA as shell were treated as controls. The different microspheres demonstrated different oxygen releasing kinetics under hypoxic conditions. The releasing of oxygen was realized because H₂O₂ was slowly and continuously released from the microspheres when the shell polymers degraded. Due to the complex formation of H₂O₂ and PVP, the releasing of H₂O₂ can last for a relatively long period of time. The released H₂O₂ was converted to oxygen by catalase. The oxygen release kinetics was measured by converting fluorescent intensity of oxygen-sensitive luminophore referring to oxygen-insensitive fluorophore to oxygen concentration with a standard curve. For the classic PLGA oxygen releasing microspheres, the microspheres released oxygen slowly in the first 3 days to 7.5% and there was a burst to 18% on Day 4. After then, the oxygen reached to the highest point on Day 5 and released sustainably in the following days at around 20%. However, hypoxia-sensitive oxygen
releasing microspheres with different NI conjugation showed very different oxygen releasing behaviors. 0NI microspheres showed lowest released kinetics at around 10%. 10NI microspheres kept releasing at around 15%. 15NI and 20NI performed faster and higher oxygen release than PLGA microspheres. For 20NI microspheres, oxygen release reached 13.4% on day 3, 24.6% on Day 4, and highest 32.6% on Day 5. Then the oxygen released from the microspheres continuously above 20% for at least 21 days. Oxygen generated from 15NI microspheres reached to the highest levels 1 day later than 20NI microspheres.

5.3.3 Fabrication catalase conjugated hypoxia-sensitive oxygen releasing microspheres and oxygen release kinetics

Further advanced catalase conjugated hypoxia-sensitive oxygen releasing microspheres were developed by H5A25N20/H5A25NI20 mixture as shell material. The surface catalase conjugated microspheres were fabricated based on amine-NHS reaction between amine group on catalase and NHS group on the H5A25N20 shell polymer. By labeling the catalase with fluorescent dye FITC, it could be observed in the fluorescent image that there was a fluorescent circle on the microsphere which indicated a successful catalase conjugation (Figure 5.10). Catalase conjugation densities on microspheres were presented in Table 5.3. The conjugation density was characterized by examining the catalase solution fluorescent intensity difference before and after conjugation. The oxygen releasing kinetics of the advanced catalase conjugated hypoxia-sensitive oxygen releasing microspheres were significantly different and largely depended on the ratio of CAT/NI as
showed in Figure 5.11. CAT/NI (100/0) microspheres were regarded as control and the oxygen releasing curve was very similar to the microspheres with non NI conjugated polymer as shell material. However, the oxygen released a little higher due to the catalase conjugation which made oxygen conversion very efficient. The conjugation density and releasing curve of CAT/NI (10/90) microspheres proved 10% catalase conjugation would be enough for catalyzing the released H$_2$O$_2$ to oxygen. The oxygen could release fast in 2 days to above 10% due to the catalase conjugated on the surface which converted H$_2$O$_2$ much more efficiently than the non-catalase conjugated microspheres. The oxygen released to the highest level on Day 5 and the microspheres could generate oxygen at high level for 28 days. CAT/NI (50/50, 30/70) microspheres showed similar release curve pattern but reduced oxygen release due to less NI on the shell materials. This indicated that more oxygen was released by the microspheres with more NI in the shell materials.

5.3.4 MSCs survival in hydrogels with hypoxia-sensitive oxygen release microspheres under ischemic condition in vitro

The efficacy of hypoxia-sensitive oxygen releasing system was evaluated by culturing MSCs in the oxygen releasing system in vitro. MSCs were mixed with hydrogel solution with addition of oxygen releasing microspheres with 20NI and 10NI conjugated polymer as shell materials and cultured in a hypoxic incubator (1% oxygen, 5%CO$_2$, 37°C) and supplied with no FBS culture medium which mimicked the ischemic conditions. The non-microspheres adding group and normal oxygen release group (using PLGA oxygen releasing microspheres) were regarded as controls for comparison.
The dsDNA content was then measured to quantify MSCs survival after 7 days culture under ischemic conditions (Figure 5.12). For the control group without adding oxygen release microspheres, the MSCs died dramatically. After 7 days, only 26.8% of MSCs survived compared to the cells number of Day 1. However, in the normal oxygen release system, there is no cell death observed on Day 3 and cell number increased to four times of the original cell numbers on Day 7. In the hypoxia-sensitive oxygen release system, a solid growth of the cell number was observed on 20NI groups. Compared to the cell number on Day 1, the number increased to 313.9% on Day 3 and 1016.8% on Day 7. For the cells in 10NI groups, no significant cell growth was observed on Day 3 but the cell number raised afterwards to 256% on Day 7. The MSCs survival results were also confirmed by live cell images (Figure 5.13).

Real-time RT-PCR was used to examine the paracrine effect at mRNA level. From the gene expression result, MSCs cultured in the hypoxia-sensitive and normal oxygen releasing system all exhibited fold increase of four paracrine factors. PDGF, HGF, IGF1 and VEGF, compared to the plate MSCs (Figure 5.14). Among them, significant gene expressions were observed on gene markers PDGFB, VEGFA and IGF1. These indicated that the survived MSCs were able to secrete paracrine factors which in turn enhanced cell survival and proliferation. Interestingly, MSCs in 20NI-ORM oxygen releasing system showed fold increase of 42 and 11 for PDGFB and IGF1 which were higher than other oxygen releasing groups.
5.3.5 MSCs survival in hydrogels with catalase conjugated hypoxia-sensitive oxygen release microspheres under ischemic condition in vitro

dsDNA contents of MSCs of each conditions at each time point were measured to identify if the hypoxia-sensitive oxygen releasing system would enhance the cell survival. From the **Figure 5.15**, dsDNA content increase was observed in CAT/NI (30/70) group and CAT/NI (10/90) group at Day 7. Significant MSCs proliferation was observed in CAT/NI (10/90) group. 227.8% cells number increase was obtained on Day 7. It was showed that in CAT/NI (30/70) group the cell number reached 140.6% on Day 7. In CAT/NI (50/50) group, there was no cell death after 7 days culture in hypoxia. However, in CAT/NI (100/0) group, very small amount of MSCs died in the culture period. 2% MSCs died on Day 3 and 8% MSCs died on Day 7. The reason of cell death was related to the insufficient oxygen release of microspheres with CAT/NI (100/0). The dsDNA results indicated that the hypoxia-sensitive oxygen releasing system with high NI component (NI ratio larger than 50%) promoted MSCs survival under ischemic conditions. The MSCs were stained with live cell tracker CM-DiL and the live cell images (as showed in **Figure 5.16**) taken by confocal microscope were consistent with the dsDNA content results.

Paracrine effect was investigated from mRNA level by real time RT-PCR. Four paracrine factors (PDGFB, HGF, IGF1, and VEGFA) were studied. Upregulated paracrine factors gene expression of experiment groups with NI/CAT ratio of 0/100, 50/50, 70/30, and 90/10 were observed. Among the four groups, the group with NI/CAT ratio of 90/10 demonstrated the highest expression of which the fold increase numbers were 20, 11, 14, and 31 for PDGFB, IGF1, HGF, and VEGFA (**Figure 5.17**).
5.4 Discussion

Previous fabricated oxygen releasing microspheres were based on slow degradation of shell material PLGA to release the enclosed PVP/H$_2$O$_2$ complex slowly to achieve sustainable oxygen release. The oxygen releasing behavior was fixed no matter how the oxygen condition was changed in the environment. And from the oxygen releasing curve, oxygen produced in the first several days was low and was insufficient to eliminate the surrounding hypoxia environment. In this work, hypoxia-sensitive polymer was synthesized and introduced to replace PLGA as new shell materials for the oxygen releasing microspheres. Fast release of oxygen in response to hypoxia was observed when the hypoxia-sensitive polymer became hydrophilic and then degraded faster under hypoxic conditions.

To fabricate the microsphere, a new shell material polymer was synthesized. The polymer should be hydrophobic and slowly biodegradable following hydrolysis degradation mechanism. NIPAAm/APLA/NAS/HEMA (50/25/20/5) was synthesized by free radical polymerization to serve as the shell material for the microspheres. Based on a previous study in our lab, APLA was a good degradable component for polymer synthesis. Thus a large percent (molar ratio 25%) of APLA monomers were introduced into the polymer to make the polymer degradable and hydrophobic as well. NAS in the polymer provided the polymer with NHS functional groups which could be used for polymer modification via simple conjugation method. It was well known that the oxygen release behavior largely depended on the degradation rate of the shell material. The degradation
rate of the polymer following hydrolysis degradation mechanism was affected by the hydrophilicity and water content of the polymer. From the previous report, the unique hypoxia responsive behavior of 2-nitroimidazole (NI) was observed when the conversion of hydrophilicity was taken place from hydrophobic component 2-nitroimidazole (NI) converted to hydrophilic 2-aminoimidazole through bioreduction triggered by hypoxic conditions. Therefore, amine-functionalized NI could be conjugated on the degradable polymer to make it hypoxia-sensitive of which the degradation rate increased when the polymer was incubated under hypoxic conditions. To investigate how the NI conjugated polymer responded to hypoxia, 20NI and 10NI conjugated polymers were successful obtained by NHS-amine conjugation. The Tg of the polymer reduced after NI conjugation because the large side group restricted the mobility of the polymer chain. More NI conjugation (20NI) had the lower Tg (45°C). From the water contact angle and water content results, the NI conjugated polymer had significantly increased water content and more affinity to water under hypoxia incubation than in normal conditions. The hypoxia responsive behavior not only depended on NI conjugation but also the amount of NI conjugated on the polymer. 20NI polymer showed higher water content and hydrophilic property than 10NI polymer. The above hypoxia-sensitive properties changes due to the amount of NI and oxygen conditions in the environment affected the polymer degradation kinetics. The degradation curves of the different amount NI conjugated polymer at normal and hypoxic conditions were consistent with the hypothesis. NI conjugated polymer degraded faster under hypoxic conditions. More NI conjugation on the polymer would made higher water affinity and water content of the polymer which led to faster degradation.
These results indicated the developed NI conjugated hypoxia-sensitive polymer is a feasible candidate for shell material of oxygen releasing microspheres.

The hypoxia-sensitive oxygen releasing microspheres were fabricated by the previously established electrospray method. The core-shell structure of the microspheres maintained as usual with the new developed hypoxia-sensitive polymer. The oxygen releasing kinetics of microspheres with different ratio of NI conjugation demonstrated how the amount of NI in shell affected oxygen releasing behavior of the microspheres under hypoxic conditions. 20NI and 15NI microspheres performed high efficient hypoxia-sensitive oxygen release compared to the PLGA oxygen releasing microspheres. The released oxygen went to a favorable level just in 3 days. This was because the shell turned to be more hydrophilic and had more water content in hypoxia and the PVP/H$_2$O$_2$ trapped in the shell materials could diffuse out quickly and further promoted fast degradation of the polymer. More complex leaked out as the polymer degraded faster and the oxygen restored to normal level in no more than 4 days (20NI ORM). The peak of oxygen generation was reached on day 5 (20NI ORM) to satisfy a high oxygen consumption scenario, for example, skeletal muscle regeneration. The amount of NI in the microspheres, which were 20NI, 15NI, and 10NI, determined the oxygen releasing rate to be high, medium, and low, respectively. The reason for different oxygen generation rate was that the degree of hydrophilicity changes of microspheres with different NI were different.

To make the microspheres more advanced and applicable in clinical use, catalase was conjugated on the surface of the microspheres to convert H$_2$O$_2$ to oxygen efficiently. Polymers H5A25N20 and H5A25NI20 were mixed and used to fabricate the microspheres.
By controlling the ratio of the two polymers, different ratios of CAT/NI could be acquired. With FITC labelling on the catalase, catalase modified microspheres could be detected by confocal microscope and the amount of catalase could also be determined. Interestingly we found that with the sequential change of CAT ratio in CAT/NI from 100/0, to 50/50, to 30/70, to 10/90, the conjugated catalase on the surface of microspheres decreased accordingly. The catalase conjugated hypoxia-sensitive oxygen releasing microspheres oxygen demonstrated efficient oxygen generation. Two bursts of oxygen release were observed from day 1 to day 2 and day 3 to day 5 which raised the oxygen amount to ideal level for use. The first quick release happened because the diffused H$_2$O$_2$/PVP met the catalase on the microsphere surface and a quick conversion to oxygen occurred. The second burst of oxygen generation was due to the release of inner contained complex.

In the cell survival in vitro study, APLA hydrogel was introduced for the cell and microspheres encapsulation because the previous study demonstrated perfect oxygen permeability of the APLA hydrogel due to its high oxygen partial pressure measured by EPR in Chapter 3. Therefore, the microspheres could respond to hypoxia environment quickly and the released oxygen in the oxygen releasing system can be efficiently used by the encapsulated cells. In Figure 5.12, the MSCs survival was promoted by encapsulating the cells into hypoxia-sensitive oxygen releasing system under ischemic culture conditions. Cell numbers were closely related to the amount of oxygen released from the microspheres. Significant cell number increase could be observed in 20NI ORM group on day 3 and day 7 compared to normal ORM and 10NI ORM. In Figure 5.15, the catalase modified
hypoxia-sensitive oxygen releasing system could enhance the MSCs survival under ischemic conditions.

5.5. Conclusion

In this work, a hypoxia-sensitive organic compound (NI) was synthesized and applied to modify a designed hydrophobic biodegradable polymer. The hydrophilic and hydrophobic properties of the organic compound NI modified polymer were proved to be sensitive to the oxygen content due to the significant difference between their water content and water contact angle in the environment. When the oxygen level changed from normal to hypoxic, the NI modified polymer converted to be hydrophilic via bioreduction process. The NI polymer was used as a new shell material for the oxygen releasing microsphere to make it hypoxia sensitive. The releasing kinetics of the microspheres was related to the amount of NI in the shell material. The fabricated oxygen releasing microspheres with shell polymer at a high NI ratio performed quick and high oxygen release. Further, catalase was also successfully conjugated onto the shell surface of the microspheres and the release of oxygen from the microspheres was proved to be similar as usual. This hypoxia-sensitive oxygen releasing microspheres were able to enhance cell survival under ischemic conditions as well as stimulate paracrine effect of the survived cells. In addition, the advanced microspheres were believed to be much more convenient and feasible when applying in hydrogel injection system to regenerate ischemia induced tissue damage by providing a quick and hypoxia responsive oxygen supply to the tissue.
Figure 5.1 Synthesis scheme of 2-(2-Nitroimidazolyl)ethanamine and bioreduction scheme of NI.
Figure 5.2 Synthesis scheme of polymer NIPAAm-co-APLA-co-NAS-co-HEMA (H5A25N20).
Figure 5.3 Synthesis scheme of NI conjugation reaction with polymer H5A25N20.
Figure 5.4 $^1$H-NMR spectrum of NI.
Figure 5.5 $^1$H-NMR spectrum of polymer NIPAAm-co-APLA-co-NAS-co-HEMA (H5A25N20).
Figure 5.6 $^1$H-NMR spectrum of NI conjugated polymer.
Table 5.1 Tg of synthesized polymers.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Tg (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H5N20A25-20Nl</td>
<td>45</td>
</tr>
<tr>
<td>H5N20A25-10Nl</td>
<td>48</td>
</tr>
<tr>
<td>H5N20A25</td>
<td>56</td>
</tr>
</tbody>
</table>
Table 5.2 Water content of 20NI and 10NI conjugated polymers under normal and hypoxia conditions.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Normal (%)</th>
<th>Hypoxic (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20NI polymer</td>
<td>89.64 ± 2.76</td>
<td>153.52 ± 19.06</td>
</tr>
<tr>
<td>10NI polymer</td>
<td>74.22 ± 4.27</td>
<td>91.86 ± 12.64</td>
</tr>
</tbody>
</table>
Table 5.3 Catalase conjugation density on the microspheres with different polymer ratio.

<table>
<thead>
<tr>
<th>Shell material</th>
<th>Polymer ratio (H5A25N20/H5A25NI20)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100/0</td>
</tr>
<tr>
<td>Conjugation Density (mg CAT/ mg ORM)</td>
<td>0.2083</td>
</tr>
</tbody>
</table>
Figure 5.7 Degradation of 20NI and 10NI conjugated polymers at normal and hypoxic conditions for 8 weeks.
Figure 5.8  A. Confocal images of core-shell structure of hypoxia-sensitive oxygen releasing microspheres. H\textsubscript{2}O\textsubscript{2}/PVP complex and NI conjugated polymer are core and shell, respectively. For imaging purpose, FITC and Rhodamine-B were added to the core and shell, respectively. B. SEM image of the hypoxia-sensitive oxygen release microspheres.
Figure 5.9 Oxygen releasing kinetics of hypoxia-sensitive oxygen releasing microspheres with different NI amount under hypoxia conditions for 28 days. PLGA microspheres were regarded as control.
Figure 5.10 Confocal image of catalase conjugation on the hypoxia-sensitive oxygen releasing microsphere surface. Catalase was labeled by FITC.
Figure 5.11 Oxygen releasing kinetics of catalase conjugated hypoxia-sensitive oxygen releasing microspheres with different NI/CAT ratio under hypoxia conditions for 28 days.
Figure 5.12 dsDNA content of MSC/Gel, MSC/Gel/ORM, MSC/Gel/20NI-ORM, and MSC/Gel/10NI-ORM cultured in hypoxia conditions for 7 days.
Figure 5.13  Live cell images of MSC/Gel, MSC/Gel/ORM, MSC/Gel/20NI-ORM, and MSC/Gel/10NI-ORM cultured in hypoxia conditions at day1, day 3 and day 7. Scale bar: 50µm.
Figure 5.14  Paracrine factors (PDGFB, IGF1, HGF, and VEGFA) gene expression of MSC/Gel, MSC/Gel/ORM, MSC/Gel/20NI-ORM, and MSC/Gel/10NI-ORM cultured in hypoxia conditions for 7 days.
Figure 5.15  dsDNA content of MSC encapsulated in hydrogel with ORM of NI/CAT ratio of 0/100, 50/50, 70/30, and 90/10 cultured in hypoxia conditions for 7 days.
Figure 5.16 Live cell images of MSC encapsulated in hydrogel with ORM of NI/CAT ratio of 0/100, 50/50, 70/30, and 90/10 cultured in hypoxia conditions at day1, day3, and day7. Scale bar: 50μm.
Figure 5.17 Paracrine factors (PDGFB, IGF1, HGF, and VEGFA) gene expression of MSC encapsulated in hydrogel with ORM of NI/CAT ratio of 0/100, 50/50, 70/30, and 90/10 cultured in hypoxia conditions for 7 days.
CHAPTER 6: Promote encapsulated mesenchymal stem cells and neural stem cells survival and differentiation with anti-inflammatory peptides functionalized hydrogels

6.1 Introduction

Stem cell therapy is considered promising tissue regeneration approach to treat cardiac injuries (heart attack), brain injuries (stroke and traumatic brain injuries) as well as ischemic limb injuries (critical limb ischemia and acute limb ischemia) [189-192]. The similarity cause of the severe diseases mentioned above is limited blood supply to the tissue which results with an ischemic environment. The low oxygen and low nutrient harsh environment lead to cell death, tissue damage and function loss in a short time. Directly delivery of stem cells into the damaged tissue was reported to be of very low efficacy due to large percent of the stem cell death during and after cell transplantation. Encapsulating stem cells into hydrogels have been proved to be an attractive therapeutic option for stem cell therapy [90, 230, 231]. The hydrogels served as delivery vehicles and extracellular matrix.

Although in previous studies as describe in Chapter 2 and 3, the survival of stem cells under ischemic conditions \textit{in vitro} and \textit{in vivo} could be improved by encapsulating cells into the hydrogel together with growth factors and oxygen releasing microspheres, these cells encountered additional challenges when they were transplanted \textit{in vivo}. When injuries
occurred, the host innate immunity responded to the necrosis of the damaged tissues and initiated normal wound healing cascades [45, 185, 232, 233]. In the healing process, inflammatory and immune cells were recruited and pro-inflammatory cytokines were secreted afterwards [233, 234]. Apoptotic pathways in the transplanted cells and host cells were then activated to lead to programmable cell death [235]. Even though the thermosensitive hydrogels developed in our lab exhibited excellent biocompatibility to the encapsulated cells [130, 131], and should protect the cells by blocking the infiltration of the large immune cells and proteins (MW>75kDa) of the immune system due to the nanoscale pore size. However, the hydrogel barriers could not prohibit the penetration of pro-inflammatory cytokines, mainly tumor necrosis factor-a (TNF-α, 17.4kDa) and interleukin-1b (IL-1β, 17kDa) [95, 236, 237]. Once these pro-inflammatory cytokines entered into the hydrogel and met the encapsulated cells, cell apoptosis pathways were quickly activated to kill the cells [235, 238-240]. Therefore, the free penetration of pro-inflammatory cytokines into the cell/hydrogel system was considered a big issue to improve transplanted cell survival [102].

The synthetic hydrogels were versatile in terms of modification and functionalization. Modifying the hydrogel surface with pro-inflammatory cytokines suppressive molecules should be a feasible way to offer the hydrogel system a function to prevent the penetration of the cytokines. Small peptides, which showed specific and high binding affinity to cytokines, were regarded as ideal options to modify the hydrogel. From TNF-α recognition loop on TNF receptor 1 (TNFR1), peptides with specific sequence was derived with the ability to recognize and bind to TNF-α [104, 241]. Peptide with similar functions to exhibit
high affinity to IL-1β was also reported [105]. Taking the advantage of the anti-inflammatory peptides, it could be hypothesized that hydrogel which was conjugated with the peptides on the surface prevented the penetration of pro-inflammatory cytokines by binding the cytokines.

In this work, an immune protection system was developed based on a functionalized thermosensitive hydrogel and specifically designed anti-pro-inflammatory cytokines peptides. The peptides were conjugated on the hydrogel surface via simple biotin-avidin bonding reaction. Biotin-avidin interaction was widely used in material modification for tissue regeneration aspects [242, 243]. This hydrogel system efficiently inhibited the binding of pro-inflammatory cytokines to cell surface cytokines receptors. Thus the pro-inflammatory cytokines mediated cell apoptosis was inhibited and cell survival was enhanced. In particular, the modified hydrogel demonstrated protective effect in protecting cell from cytokines attack and enhancing cell survival under TNF-α and IL-1β conditions. The strategy of functionalizing hydrogels with anti-inflammatory peptides could be considered potentially applicable in the tissue regeneration clinically.

6.2 Materials and methods

6.2.1 Materials

N-isopropylacrylamide (NIPAAM, TCI) was recrystallized with hexane for three times before use. 2-Hydroxyethyl methacrylate (HEMA, Alfa Aesar), DL-α-hydroxy-β,β-dimethyl-γ-butyrolactone (Sigma), trimethylamine (Sigma), acryloyl chloride (Sigma),
biotin hydrazide (Sigma), and streptavidin (Sigma) were used as received. Two peptides were ordered from Celtek Bioscience, LLC. Recombinant mouse IL-1β and TNF-α were purchased from Peprotech.

6.2.2 Dimethyl-γ-butyrolactone acrylate (DBA) synthesis

20g DL-α-hydroxy-β,β-dimethyl-γ-butyrolactone and 38.4mL TEA were mixed in 200mL dichloromethane in a 500mL round bottom flask and the mixture was stirred on ice for 30 minutes. 14.93mL acryloyl chloride was carefully added dropwise to the mixture. After 3 hours, the mixture was filtered and the filtrate was washed with 50mL dichloromethane. The filtered solution was washed with DI water in a separation funnel for at least 3 times. The organic phase was collected and dried with MgSO₄. The mixture was vacuum filtered and the organic phase was collected. After the solvent was removed, DBA was obtained and kept in -20°C freezer before use.

6.2.3 N-acryloxysuccinimide (NAS) synthesis

11.5g N-hydroxysuccinimide and 15.15mL trimethylamine were dissolved in 150mL chloroform at 0°C. 10g acryloyl chloride was added dropwise to the mixture. After the mixture was stirred on ice for 40 minutes, the mixture was washed sequentially with 80mL ice cold water and saturated brine. Then the organic phase was collected, dried with MgSO₄ and filtered. 5mg 2,6-di-tert-butyl-4-methylphenol was added to the solution and then the solution was concentrated to a volume of 30mL using a rotary evaporator followed by
filtration. 3mL ethyl acetate and 20mL hexane were mixed and added slowly with stirring to the chloroform solution. The solution was left steady at 0°C for several hours for crystallization. The colorless crystals were separated by vacuum filtration and the crystals were washed sequentially with ice cold 10mL hexane/ethyl acetate (4/1), 10mL hexane/ethyl acetate (9/1), 20mL hexane. Then the crystals were dried in vacuum at ambient temperature to constant weight and stored in -20°C freezer before use.

6.2.4 Hydrogel polymer synthesis and properties characterization

The hydrogel was synthesized by copolymerizing NIPAAm, DBA, NAS and HEMA, using free radical polymerization method as showed in Figure 6.1. NIPAAm (4.18g), DBA (0.36g), NAS (0.82g) and HEMA (0.64g) were dissolved in dioxane at a specific molar feeding ratio (76/4/10/10) in a 250mL, three-neck round bottom flask. The initiator benzoyl peroxide (BPO, 24mg) was added and the polymerization was conducted in 70°C oil bath with nitrogen protection overnight. The polymer was precipitated out by pouring the solution into hexane with stirring. The filtered polymer was further purified twice by dissolving in tetrahydrofuran (THF) and precipitating in ice cold ethyl ether. The obtained polymer was vacuum dried overnight before use. To biotinylate the hydrogel polymer (Figure 6.2), 1g polymer and 0.1g biotin hydrazide were dissolved in 40mL DMF with addition of 56μL trimethylamine. The reaction was conducted under the protection of nitrogen at around 60°C overnight. After the solvent DMF was eliminated, the brown product was dissolved in 10-15mL THF and precipitated in anhydrous ethyl ether three
times. The precipitate polymer was filtered and vacuum dried before use. The structure and ratio of components were confirmed by $^1$H-NMR.

Hydrogel solutions in this study were prepared by dissolving the hydrogel polymer in Dulbecco’s modified phosphate buffer saline (DPBS, pH = 7.4) at a concentration of 20% (w/v). The thermal transition temperature of the solution was measured by using differential scanning calorimetry (DSC) over a temperature range of 0-60°C, with a heating rate of 10°C/min. The gelation time of the hydrogel solution at 37°C was measured using an Olympus IX771 microscope equipped with a temperature control chamber of 37°C. The hydrogel solution was dropped onto a pre-warmed glass slide on the microscope stage. A video was recorded and the gelation time was determined when the transmittance of the hydrogel solution from clear to opaque was observed.

The solid hydrogel was acquired after the hydrogel solution was gelled in a 37°C water bath. After several hours to reach equilibrium water content, the hydrogel was ready for water content and mechanical test. The wet weight of the hydrogel was measured as $w_1$, while the lyophilized hydrogel weight was measured as $w_2$. The water content was calculated as: Water content (%) = ($w_1$-$w_2$)/$w_2$×100%

The mechanical properties of the hydrogel were tested with an Instron tensile tester, using a cross-head speed of 50 mm/min. The hydrogel modulus was calculated from the elastic deformation region of the stress-strain curves, using a MATLAB program.

To determine hydrogel degradation, 200μL of hydrogel solution were added to a 1.5mL microcentrifuge tubes and the tubes were placed in a 37°C water bath. After incubation for several hours, the supernatant was replaced with 200μL DPBS. The
degradation was conducted for 8 weeks at 37°C. The samples were taken at each time point and weight of hydrogel was measured as $w_3$ after they were freeze-dried. The weight of sample before degradation (week 0) was $w_4$. The weight remaining was calculated as:

$$\text{Weight remaining (\%) } = \frac{w_3}{w_4} \times 100\%$$

6.2.5 Peptide conjugation on the hydrogel and conjugation density measurement

Peptide (FITC labeled) solutions were prepared by dissolving peptide powder into DPBS to final concentrations of 0.25, 0.50, 1.00, 2.00mg/mL. The peptide was conjugated on the hydrogel surface via biotin-avidin conjugation method as demonstrated in Figure 6.3. The hydrogel solutions were first allowed to solidify under 37°C for 30 minutes in a glass vial. The solid hydrogels were pouch to disks with a 6mm pouch and the hydrogel disks (~1mm thickness) were incubated in 37°C DPBS before transferring to 48 well plate. 1mL warm DPBS and 10µL 1mg/mL streptavidin solution was added to each well of 48 well plate and the plate was incubated under 37°C for 20 minutes. Then the solutions in the wells were removed. After the disk was washed with DPBS 3 times to remove the unreacted streptavidin, 1mL peptide solution (0.25, 0.50, 1.00, 2.00mg/mL) was added to each well. The plate was incubated under 37°C for 10 minutes with occasionally shaking (every 2 minutes) and the disks in the plate was turned upset down. After another 10 minutes incubation, the disk in each well was washed 3 times with DPBS. To fabricate both peptides conjugated hydrogel, solutions containing two peptides at concentrations of 0.25 and 1.00mg/mL were prepared. And the conjugation procedure was the same as previously describe by using the newly prepared solutions containing two peptides.
To determine the peptide conjugation density, the disk was placed in 96 well black plate with addition of 190μL DPBS. The plate was kept in 4°C to allow gel-solution transition and then read the fluorescent intensity of FITC by using fluorescent plate reader. The amount of peptide was calculated by converting the fluorescent intensity number to peptide concentration via intensity - concentration standard calibration curve.

6.2.6 Mesenchymal stem cell and neural stem cell culture

Mouse bone marrow-derived mesenchymal stem cells (MSCs) were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) with 10% fetal bovine serum (FBS) and 1% penicillin at 37°C with 5% CO₂ and 21% O₂. Medium was changed every three days. Cells were passaged when reached 90% confluences. Cells at passages 14 - 20 were used in this experiment. Our previous works demonstrated that bmMSCs within these passages remained multipotency.

Mouse neural stem cells (NSCs) were isolated from mouse intestines tissues. The enteric neural stem cell isolation procedure was described in Chapter 3. The obtained cells were cultured in 35 mm Petri dishes in DMEM/F12 medium containing 100 U/ml penicillin and 100μg/ml streptomycin (Invitrogen), supplemented with 2mmol/L L-glutamine (Invitrogen), 7.5% (v/v) chick embryo extract (Gemini Bio-products), 1% (v/v) N2 medium supplement (Sigma-Aldrich), 20ng/mL mouse basic fibroblast growth factor, and 20ng/mL mouse epidermal growth factor (Sigma). Medium was changed every four days. NSCs grew as free-floating cellular aggregates known as neurosphere-like bodies. Cell passage was proceeded at 90-95% confluence. Cells were dissociated into single cells.
and subculture again. Cells within passage 7 were used in this experiment to make sure the multipotency of the NSCs.

6.2.7 Cells encapsulation into hydrogel

20% (w/v % in DPBS) hydrogel solution was used to encapsulate MSCs and. MSCs were digested from the tissue culture plates, centrifuged to acquire cells pellet and re-suspended cells in culture media. Before encapsulation, hydrogel solution was sterilized under UV light in a laminar flow hood for 30 minutes. Cell suspension was then added into the hydrogel solution to a final cell concentration of 4 million/mL. After gelation of the hydrogel, peptide modification was proceed using the previous described method. The cell encapsulation did not affect peptide conjugation. The modified Hydrogel/Cell disks were cultured with medium added IL-1β and TNF-α (20ng/mL) under normal culture condition (10% FBS, 21% O₂, 5% CO₂). Hydrogel/Cell culture in normal culture medium was used as a control. At day 1, 3 and 7, disks were randomly picked for dsDNA content measurement, live cell observation, real time RT-PCR test and immunohistology analysis.

6.2.8 In vitro cells culture in anti-inflammatory hydrogel under inflammatory conditions

The survival of MSCs and NSCs in hydrogel with or without peptide modification was characterized by double-strand DNA (dsDNA, for live cells) content. Samples collected at day 1, 3 and 7 were washed with DPBS for 3 times and then treated with papain solution and incubated under 60°C overnight. The dsDNA concentration in the digested papain
solution was then measured by PicoGreen assay (Invitrogen) following the protocol provided by the kit, and normalized to the dry weight of the hydrogel polymer.

As for the differentiation of the encapsulated cells in hydrogels, real time RT-PCR and immunohistology were used to detect MSC and NSC gene and protein expressions, respectively. After 7 days in vitro culture, hydrogel/MSC disks with or without peptide protection were immersed in TRIzol (Sigma) and processed following the manufacture's protocol to isolate and purify RNA. The amount of extract RNA was quantified by Nanodrop (Thermo). cDNA was reverse transcript from RNA by using cDNA high capacity transcription kit (ABI). A real-time RT-PCR quantification was conducted by using SYBR Green/fluorescein (Fermentas) following the provided method. Markers GFAP, NESTIN and MAP2 were checked and β-actin was used as the housekeeping gene. A standard ΔΔCt method was used to process the data. For protein expression, the hydrogel samples were first fixed in 4% paraformaldehyde for 1 h and then washed with PBS. The fixed samples were embedded in OCT (Tissue-Tek) and frozen sectioned at 5µm. The sections were blocked by 10% goat serum blocking buffer with 0.3% Triton X-100 at 37°C for 1h. Immunohistological staining for GFAP, NESTIN, and MAP2 were performed on the tissue sections on the slides stained with primary antibody accordingly overnight at 37°C in a humid chamber. Secondary antibody was then conjugated to the primary antibody at room temperature for 1h. Nucleus was stained with DAPI. Negative control sections were prepared by the same procedure, but without the primary antibody incubation. The slides were observed and images were taken with FV2000 filter confocal microscope.
6.2.9 In vivo implantation of anti-inflammatory MSCs delivery system into mouse ischemic limb

MSCs were encapsulated into the hydrogel and then the Gel/MSC system was modified with anti-inflammation peptides following the previously described method. The Male wild type mice (aged 8 to 10 weeks) were anesthetized with an i.p. injection of ketamine 80 mg/Kg and xylazine 5 mg/Kg before all surgical procedures. Hind limb ischemia was induced by unilateral femoral artery and vein ligation, as indicated in Figure 6.4. After the vessel ligation, one 6mm Gel/MSC disk were implanted into the muscle gap in the affected tissue area between the ligation points of the ischemic mice limbs. Gel/MSC implantation group were used as control. MSCs were labelled with live cell tracker. The mice were raised for 14 days before sacrificed. The muscles were collected and fixed in 4% paraformaldehyde overnight at 37°C. The muscles were then proceeded with paraffin embedding and sectioning. Fluorescence images were taken with the paraffin tissue sectioning slides by Olympus FV1000 filter confocal microscope, and survived cell numbers/densities were analyzed based on these images. Immunohistological staining for Ki67 (Abcam), Nestin (Abcam), MHC (Millipore) and vWF (Millipore) were performed on the tissue sections on the slides following the provided standard approaches. The nucleus was stained with DAPI. The slides were observed with FV2000 filter confocal microscope and the confocal images were used to evaluate cell proliferation, differentiation, and blood vessel formation, respectively.
6.2.10 Statistical methods

Data are expressed as mean ± standard deviation. Statistical comparisons were performed by ANOVA using JMP. Significant difference is defined as p < 0.05.

6.3 Results

6.3.1 Polymer synthesis and hydrogel characterizations

Before polymerizing the hydrogel polymer, two functional monomers were synthesized. The DBA served as degradable component of which the degradation product was believed to dissolve in DPBS at 37°C. NAS provided the polymer with a functional side group for modification. The NHS group from NAS component was able to react with amine group via NHS-amine conjugation.

The biodegradable thermosensitive hydrogel polymer was synthesized by copolymerization of NIPAAm, DBA, NAS and HEMA using free radical polymerization method with four feed ratios. The biotinylated polymer was synthesized by conjugating biotin hydrazide on the H10N10D4 or H10N15D4 polymer via simple amine-NHS reactions. The structures of the synthesized hydrogel polymers and biotinylated polymers were confirmed by $^1$H-NMR spectrum (Figure 6.5 and Figure 6.6). The molecular ratio of NIPAAm/DBA/NAS/HEMA and the amount of biotin group conjugated on the polymer were consistent with the feed ratios which were demonstrated in Table 6.1 and Table 6.2. Sol-gel transition temperature of the hydrogel H10N10D4 of 20% solutions were determined to be 26.1°C. Therefore, the 20wt% hydrogel solution was in liquid form and
flowable at 4°C. It was able to inject through a 26-gauge needle which is usually used in surgery of tissue for cells injection (Figure 6.7). The hydrogel was able to solidify under 37°C in 7 second. The short gelation time helped improve cell retention rate after implantation. After equilibrium the gel solution in 37°C, the soft gel had a water content of 86.86 ± 18.88 %. Previous studies reported that the mechanical properties of the hydrogel could affect the cell fate. To figure out a proper concentration of hydrogel solution to be applied for cell encapsulation, mechanical properties of 5%, 10% and 20% hydrogel solutions were measured and showed in Table 6.3. The hydrogels showed highly flexibility with Young’s modulus decreasing with the decrease of concentration. 20% hydrogel possessed a modulus of 60.09 ± 3.44 kPa while 5% hydrogel possessed a modulus of 9.97 ± 0.59 kPa. When the H10N10D4 and H10N15D4 hydrogels were incubated at 37°C in DPBS, they degraded slowly and had weight remaining of 81.9% and 87.9% after 8 weeks (Figure 6.8). The degradation product was soluble at 37°C because the thermal transition temperature of degraded hydrogel raised above 37°C. The hydrogel demonstrated high oxygen permeability in EPR testing. The oxygen permeability of H10N10D4 hydrogel was 115.2mmHg while oxygen partial pressure at room air condition was 160mmHg.

6.3.2 Anti-inflammation peptides conjugation on the hydrogel polymers

The anti-TNF-α and anti-IL-1β peptides were specially designed with FITC labelled and biotin group at one end for conjugation reaction. Biotin-avidin conjugation technique was used to conjugate peptides on the biotinylated hydrogel polymer. The conjugation density was quantified by FITC fluorescent intensity calibration curve. To evaluate the
conjugation efficiency, hydrogel polymers with two different biotin ratio and peptides solution of three different concentrations were used (Table 6.4). From the table, we found that peptides conjugation density increased with the increasing concentration of peptide solutions as well as the molar ratio of biotin side groups in the hydrogel polymer. Even though the conjugation density increased with the increase in peptide solution concentration, it was obviously not a linear relationship. The binding density of anti-IL-1β peptide was lower than the anti-TNF-α peptide on the polymer at same condition, respectively. For later application, conjugation reaction combination of 1.00mg/mL peptide concentration and H10N5B5D4 polymer was chosen. And the conjugation densities were \((5.46 \pm 0.47) \times 10^{-5}\) and \((3.79 \pm 0.43) \times 10^{-5}\) mg/mm\(^2\) for anti-TNF-α and anti-IL-1β peptide.

6.3.3 *In vitro* NSCs culture in anti-inflammation hydrogels under inflammation conditions

To determine a proper hydrogel solution concentration to be used for the anti-inflammation system, hydrogel solutions with 3 different concentrations were used for NSCs culture *in vitro* under normal culture conditions. The primary thing is the cell proliferation ability. dsDNA content result demonstrated that after 7 days cell culture, NSCs in 20% and 10% hydrogel solution showed significantly higher proliferation ability than NSCs in the 5% hydrogel solution (Figure 6.9). In addition, NSCs cultured in 20% hydrogel had highest MAP2 fold increase than NSCs in 10% hydrogel solution (Figure 6.10). Thus, 20% hydrogel solution was selected for *in vitro* and *in vivo* investment.
In the following *in vitro* study, NSCs were cultured separately in anti-TNF-α hydrogel system, anti-IL-1β hydrogel system and anti TNF-α & IL-1β hydrogel system. In TNF-α conditions, NSCs died dramatically in hydrogel without peptide conjugation (*Figure 6.11*). Only around 45% cells left after culture in the medium with pro-inflammatory cytokines TNF-α for 7 days. However, NSCs were able to survive and proliferate in anti-TNF-α hydrogel system. More NSCs were found in high peptide conjugation hydrogel group. After 7 days cell culture, the cell number increased to more than 4 times the original cell number. In IL-1β conditions, NSCs died dramatically in hydrogel without peptide conjugation. Only around 42.4% cells left after culture in the medium with pro-inflammatory cytokines IL-1β for 7 days (*Figure 6.14*). However, anti-IL-1β hydrogel system promoted NSCs survival and proliferate in the harsh environment. No cell death was observed on day 3 for both high and low peptide conjugation groups. More NSCs were found in high peptide conjugation hydrogel group. After 7 days cell culture, the cell number doubled. In inflammation conditions with two pro-inflammatory cytokines presence, large amount of NSCs died in hydrogel without peptide conjugation in a short time (*Figure 6.17*). Only around 33.6% cells left after on Day 3 and 19.5% on Day 7. However, NSCs were able to survive and proliferate in anti-inflammation hydrogel system with two peptides conjugation. NSCs survival was greatly enhanced in the peptides conjugation hydrogel. After 7 days cell culture, the cell number significantly increased and it was found that the proliferation ability of NSC was higher in hydrogel system with higher peptides conjugation density. The live cell images of each *in vitro* experiment groups under different conditions were consistent with the dsDNA content results.
Real-time RT-PCR was used to examine the NSC differentiation at mRNA level. From the gene expression result, NSCs cultured in hydrogel could differentiate into neural cells. Even though, inflammation conditions dramatically depressed the NSCs differentiation. In pure hydrogel group, low gene expression was observed for genes GFAP, NESTIN and MAP2. In contrast, in anti-inflammation hydrogel systems, there were significant fold increase of NSCs observed in the investigated three differentiation gene markers. With higher peptide conjugation density on the hydrogel, the neural differentiation of NSCs in the anti-inflammation hydrogel system was promoted. For high peptide conjugation conditions of anti-TNF-α hydrogel system (Figure 6.12), the neural differentiation was upregulated to 40, 942, and 756 times for GFAP, NESTIN and MAP2. For high peptide conjugation conditions of anti-IL-1β hydrogel system (Figure 6.15), the neural differentiation was upregulated to 10, 19, and 30 for GFAP, NESTIN and MAP2. For high peptide conjugation conditions of anti TNF-α & IL-1β hydrogel system (Figure 6.18), the neural differentiation was upregulated to 79, 107, and 23 for GFAP, NESTIN and MAP2. In addition, the neural differentiation of the NSCs were also evaluated at protein level by immunohistochemistry. The staining results demonstrated that anti-inflammation peptides conjugated hydrogel system promoted NSCs neural differentiation due to the fact that the NSCs encapsulated in anti-inflammation hydrogel expressed the differentiation markers (Figure 6.13, Figure 6.16, and Figure 6.19).
6.3.4 In vitro MSCs culture in anti-inflammation hydrogels under inflammation conditions

To determine a proper hydrogel solution concentration to be used for the anti-inflammation system, hydrogel solutions with 3 different concentrations were used for MSCs culture in vitro under normal culture conditions. dsDNA content result demonstrated that after 7 days cell culture, MSCs in 20% hydrogel solution showed significantly higher proliferation ability than MSCs in the other two hydrogel solutions (Figure 6.20). In addition, MSCs cultured in 20% hydrogel expressed neural differentiation gene with fold increase compared to the plate MSCs (Figure 6.21). Thus, 20% hydrogel solution was selected for in vitro and in vivo investment.

In the following in vitro study, MSCs were cultured separately in anti-TNF-α hydrogel system, anti-IL-1β hydrogel system and anti-TNF-α & IL-1β hydrogel system. In the three conditions, MSCs died dramatically in hydrogel without peptide conjugation. Only around 20% cells left after culture in the medium with pro-inflammatory cytokines for 7 days. It was found that more peptide conjugated on the hydrogel surface led to enhanced cell survival under inflammation conditions. For anti-TNF-α hydrogel system (Figure 6.22), MSCs survival was enhanced on Day 3 with the peptide protection. On Day 7, cell number largely increased and the proliferation ability of MSCs were proportional to the peptide conjugation density. In high peptide solution conjugation condition, the MSCs number almost recovered to the normal culture conditions. For anti-IL-1β hydrogel system (Figure 6.27), MSCs survival was enhanced on Day 3 as the cell numbers were similar to the MSCs under normal culture conditions. On Day 7, there was no significant difference between
cell numbers in low amount peptide conjugation and medium amount peptide conjugation conditions. However, in high amount peptide conjugation condition, the MSCs proliferation was significantly promoted. For two peptides conjugation system (Figure 6.32), MSCs survival was significantly enhanced on Day 3 and Day 7. Cell numbers in high amount peptide conjugation even in recovery to normal status as the cell numbers were similar to the MSCs under normal culture conditions at day 7.

The differentiation of MSCs in anti-inflammation hydrogel system was characterized in gene and protein level by measuring the neural differentiation gene marker fold increase via real time RT-PCR and performing immunohistological staining of antibodies (GFAP, NESTIN, and MAP2) on the fixed sample sections. In TNF-α conditions, MSCs differentiation was suppressed as very low fold increase of gene expressions were detected. However, in anti-TNF-α hydrogel system, the differentiation of MSCs was significantly enhanced. In the RT-PCR result (Figure 6.23), fold increase of 451, 96 and 363 for gene marker GFAP, NESTIN and MAP2 respectively was demonstrated by MSCs in high anti-TNF-α peptide conjugating hydrogel system. In IL-1β conditions (Figure 6.28), MSCs in anti-IL-1β hydrogel system showed higher upregulated differentiation gene expression than MSCs without peptide protection. In the high peptide conjugated hydrogel, MSCs expressed significant fold increase of 54, 181, and 326 times for neural differentiation marker GFAP, NESTIN, and MAP2 respectively. In the mimic inflammation conditions with both pro-inflammatory cytokines presence in the culture medium (Figure 6.33), two peptides conjugated hydrogel system demonstrated the ability to promote MSC differentiation. Even in low peptides conjugation hydrogel, MSC differentiation was
restored more than 3 times to the MSC in pure hydrogel. In regarding with plate MSCs (the control), MSCs which were cultured in hydrogel with high peptides conjugation density demonstrated significant fold increase of 889, 232, and 211 for GFAP, NESTIN, and MAP2 respectively. In addition, confocal images of IHC staining of MSCs fixed and sectioning slides with GFAP, NESTIN, and MAP2 reflected the differentiation protein expression of the cells. The immunohistochemistry results (Figure 6.24-26, Figure 6.29-31, and Figure 6.34-36) were consistent with the gene expression results. MSCs in the anti-inflammation hydrogel system demonstrated intense differentiation markers in the confocal images.

6.3.5 In vivo MSCs survival and proliferation in anti-inflammation hydrogel delivery system

The animal model for experiment is mouse ischemic limb model. Ligations were made as showed in Figure 6.4 to cause limb ischemia formation and muscle tissue damage. MSCs encapsulated anti-inflammation hydrogel system was then implanted into the ischemic tissue site. Before encapsulated into the hydrogel system, MSCs were labelled with long term live cell tracker CM-DiL for live cell investigation. After 2 weeks, the tissues were collected, fixed with 4% paraformaldehyde and sent for paraffin sectioning. Proliferation marker Ki67 primary antibody was applied to staining the slides to label the cells which possessed proliferation potency. Confocal images were taken as showed in Figure 6.37. The live cell number in each conditions were calculated based on at least 4 images. The statistic live cell number results indicated that under ischemic environment
with inflammation, single peptide conjugated hydrogel system helped the implanted MSCs survival at some degree while two peptides conjugated hydrogel system significantly enhanced MSCs survival compared to the control – MSC/Gel implantation (Figure 6.37). Compared to the MSC/Gel survived cell density which was 519 cells/mm², the survived cell density reached 1013 cells/mm² in two peptides conjugated hydrogel group. Confocal images demonstrated that partial implanted MSCs and some host tissue cells were Ki67 positive (Figure 6.38). Ki67 positive MSCs density were 132, 71, and 143 cells/mm² for anti-TNF-α hydrogel system (Figure 6.40), anti-IL-1β hydrogel system and anti TNF-α & IL-1β hydrogel system which were significantly higher than the control with cell density of 50 cells/mm². Total Ki67 positive cells number (MSCs and host cells) were doubled in peptides conjugated hydrogel system than in non-modified hydrogel.

6.3.6 In vivo MSCs differentiation in anti-inflammation hydrogel delivery system and vascularization in tissue

Myogenic differentiation and neural differentiation of MSCs were characterized by immunohistological staining of primary antibody MHC and NESTIN. As Figure 6.39 showed, some implanted MSCs were able to integrate into host tissue and differentiated into skeletal muscle which were MHC positive while some cells differentiated into endothelial cells and took part in blood vessels formation as indicated by vWF staining. Partial MSCs demonstrated neural differentiation since the expression of NESTIN positive cells were detected (Figure 6.38). Based on the IHC slides, the differentiation of MSCs were characterized quantitatively by counting the differentiated cell numbers. In Figure
more MHC positive cells were observed in peptide conjugation groups than the MSC/Gel group. In single peptide conjugation group, no significant difference of myogenic differentiated cells was detected as the MHC positive cell densities were all around 174 cells/mm². By modifying the hydrogel with both peptides, myogenic differentiation was promoted as 206 cells/mm² MHC positive cells were detected. Similarly, as demonstrated in Figure 6.42, more NESTIN positive cells including MSCs and host cells were observed in anti-TNF-α hydrogel system (96.8 cells/mm²), anti-IL-1β hydrogel system (75.3 cells/mm²), and anti-TNF-α & IL-1β hydrogel system (258.1 cells/mm²) compared to no peptide hydrogel system (32.3 cells/mm²). By comparing the number of blood vessels in each experiment groups, a significant increase of blood vessel numbers was found when anti-TNF-α & IL-1β peptides were used together in modifying the hydrogel to protect the encapsulated MSCs and eliminate the inflammation effect. In Figure 6.43, 3 times of blood vessels, as indicated by vWF, were observed in 2 peptides conjugation hydrogel system than in no peptide conjugation hydrogel system.

6.4 Discussion

Immune response took place in many ischemia disease including myocardial infarction, brain trauma, and limb ischemia. Normal regeneration approach of implanting biomaterials and cells together into the target tissue area confront with big challenges of infiltration of small pro-inflammatory cytokines (TNF-α and IL-1β) which induced cell apoptosis. This issue caused dramatic cell death after implantation and therefore led to low therapeutic regeneration efficacy. In this work, elimination of immune attack from small pro-
inflammatory cytokines, mainly TNF-α and IL-1β, were investigated. Peptides which could specifically bind to the cytokines were reported. Two peptides were designed based on previous reports and the peptides were also functionalized with biotin group to allow conjugation reaction. The anti-inflammation hydrogel system was fabricated based on conjugating the peptides on a newly developed biodegradable thermosensitive hydrogel via biotin-avidin conjugation method. The anti-inflammation hydrogel system enhanced encapsulated MSCs survival, proliferation and differentiation \textit{in vitro} and \textit{in vivo}.

The NIPAAm based thermal sensitive hydrogel was developed to acquire a series of properties which were favorable for clinical implantation. The hydrogel solution had a gelation temperature of around 26.1°C. The operations of mixing the stem cells and hydrogel solution at 4°C were feasible. The slow degradation of the hydrogel provided long time protection of the encapsulated cells from the immune attack during the regeneration process while also allowing migration of cells to integrate into the host tissue and differentiation.

Peptide conjugated hydrogel was made via biotin-avidin conjugation which was an efficient reaction method for surface modification. There was four binding site in streptavidin. When it was binding on the hydrogel, 3 sites were left for peptides to conjugate on. This meant that, with less functional group in the hydrogel, large amount of peptides was able to conjugate on the hydrogel to form a high peptide density hydrogel surface. \textbf{Table 6.4} demonstrated that the conjugation degree increased when more biotin groups were on the hydrogel polymer. And ideally the conjugation degree should be doubled in H10N15B10D4 with 2 times biotin groups. This was because steric hindrance
restricted peptides conjugation when more biotin groups were on the hydrogel. The hydrogel system with peptide shielding barrier should prevent small cytokines penetration and therefore provided immune protection of encapsulated cells.

*In vitro* cell culture of MSCs and NSCs results demonstrated that the cell fate was significantly related to the anti-inflammation hydrogel system and the peptide conjugation density on the hydrogel surface. Even though hydrogel could serve as a barrier to block the infiltration of large immune cells and proteins, significant cell death in pure hydrogel delivery system was observed. The major cell death was a result of cell apoptosis which was induced by the small pro-inflammatory cytokines TNF-α and IL-1β. Generally, the cell survival rate was improved with increasing peptide conjugation density. Even the low peptide conjugated hydrogel system could ensure no cell death in short period of time for both MSCs and NSCs. This owed to the high cytokine binding affinity of the peptide on the hydrogel surface which greatly prevent the penetration of TNF-α and IL-1β into the hydrogel. In hydrogel system with high peptide conjugated density, the cell proliferation ability recovered to normal culture conditions. This indicated that the inflammation effect was eliminated since almost no pro-inflammatory cytokines could pass through the shield of peptide to trigger cell apoptosis.

The differentiation of MSCs and NSCs in anti-inflammation hydrogel was promoted due to two major reasons. Mechanical properties of cell culture matrix could influence cell fate. It was reported that biomaterial matrix with similar modulus to the target regeneration tissue stimulated stem cells differentiation into the target tissue cell types [132, 155, 244]. For example, NSCs cultured in soft hydrogel with modulus similar to brain tissue
demonstrated high neural differentiation ability. Hydrogel solution at different concentration formed hydrogel with different modulus. Hydrogels with series modulus were applied to investigate how the two stem cells proliferate and differentiate in them. For both cases, 20% hydrogel demonstrated highest cell proliferation and considerable differentiation potency. Another reason related to MSCs and NSCs differentiation regarding to the upregulated neural gene markers was the cell survival rate. With more survived cells in high peptide conjugation hydrogel system, the differentiation was promoted since more cell-cell and cell-host tissue interaction could in turn stimulate cell differentiation.

Limb ischemia was one kind of vascular diseases which restricted mobility of patients. After the happen of ischemia, local ischemic environment causes cell death and muscle degeneration, followed by the loss of tissue structure and function. The survival and functional maintenance is of especially great importance before the buildup of new blood vessels. Injection of stem cells with biomaterial matrix together with biomolecules like growth factors (in Chapter 2) or oxygen delivery systems like oxygen releasing microspheres (in Chapter 3) could augment cell survival under ischemic conditions. However, the immune responsive inflammation reaction accompanied with ischemia which led dramatically cell death had not been well addressed. The in vivo results demonstrated that by encapsulating MSCs in anti-inflammation hydrogel system and implanting into the ischemic tissue area, MSCs survival was significantly enhanced compared to the MSC/Gel group because major pro-inflammatory cytokines were blocked outside of the cell delivery system. The survived MSCs were able to proliferate and migrate
into host tissue. In addition, myogenic differentiation of MSCs was observed in gel and in host tissue. The differentiated MSCs in host tissue were expected to form aligned structures along with the native tissue pattern and then they should finally form myofibers integrated with the host tissue and functioning properly. Besides, the MSCs were also able to differentiate into endothelial cells as indicated in Figure 6.39. More blood vessels formation was observed in anti- TNF-α and IL-1β hydrogel groups. This can be a potential result of paracrine effect of the survived MSCs which was stimulated in ischemia. With more MSCs survival and proliferation, they secreted paracrine factors including angiogenesis growth factor like VEGF and bFGF and therefore promoted the MSCs endothelial differentiation and blood vessel formation.

6.5 Conclusion

In conclusion, we developed an immunoprotective hydrogel system to successfully prevent the penetration of pro-inflammatory cytokines and therefore enhanced the encapsulated cells survival under harsh environment with immune rejection in vitro and in vivo. The fabricated immunoprotective hydrogel was conjugated with anti-inflammatory peptides on the surface which demonstrated high binding affinity to the pro-inflammatory cytokines TNF-α and IL-1β. The protective effect of the system related with the conjugation degree of the peptides on the hydrogel. The survival rate of the encapsulated stem cells was significantly increased by conjugating both peptides as well as the oxygen releasing microspheres in vitro and in vivo. The encapsulated NSCs and MSCs also showed neural differentiation properties in the hydrogel of selected concentration and the
differentiation was promoted in the anti-inflammation peptides conjugated hydrogel system *in vitro* and *in vivo*.

This immunoprotective hydrogel system was proved to be an efficient stem cell delivery system and therefore offered an alternative therapeutic approach for the stem therapy to overcome the issue of inflammation effect on the transplanted cells in tissue regeneration process.
Table 6.1  Hydrogel polymer composition ratio, LCST, water content and injectability.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Feed ratio (NIPAAm/DBA/NAS/HEMA)</th>
<th>Component ratio</th>
<th>LCST (°C)</th>
<th>Water content (%)</th>
<th>Injectability</th>
</tr>
</thead>
<tbody>
<tr>
<td>H10N10D4</td>
<td>76/4/10/10</td>
<td>76/3.75/9.02/9.00</td>
<td>26.1</td>
<td>86.86±18.88</td>
<td>+</td>
</tr>
<tr>
<td>H10N15D4</td>
<td>71/4/15/10</td>
<td>71/3.86/14.74/7.08</td>
<td>25.8</td>
<td>116.42±4.17</td>
<td>+</td>
</tr>
<tr>
<td>H15N10D4</td>
<td>71/4/10/15</td>
<td>71/3.83/9.70/15.47</td>
<td>25.6</td>
<td>153.84±30.20</td>
<td>+</td>
</tr>
<tr>
<td>H20N10D4</td>
<td>66/4/10/20</td>
<td>66/3.50/9.76/18.36</td>
<td>23.5</td>
<td>115.70±21.84</td>
<td>+</td>
</tr>
</tbody>
</table>
Table 6.2  Hydrogel polymer composition ratio of biotinylated polymer.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Expect Ratio of biotin hydrazide and NAS</th>
<th>Ratio After Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>H10N5B5D4</td>
<td>5/5</td>
<td>4.97/5.03</td>
</tr>
<tr>
<td>H10N5B10D4</td>
<td>10/5</td>
<td>7.20/7.80</td>
</tr>
</tbody>
</table>
Table 6.3  Modulus and tensile strength of 5%, 10%, and 20% hydrogels.

<table>
<thead>
<tr>
<th>Hydrogel concentration</th>
<th>Modulus (kPa)</th>
<th>Tensile strength (kPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20%</td>
<td>60.09±3.44</td>
<td>21.86±6.48</td>
</tr>
<tr>
<td>10%</td>
<td>30.94±1.53</td>
<td>21.01±8.86</td>
</tr>
<tr>
<td>5%</td>
<td>9.97±0.59</td>
<td>10.53±1.89</td>
</tr>
</tbody>
</table>
Table 6.4  Anti TNF-α and anti IL-1β peptides conjugation density on the hydrogel.

<table>
<thead>
<tr>
<th>NIPAAm-DBA-NAS-(NAS-biotin)-HEMA Hydrogel</th>
<th>Anti TNF-α peptide concentration(mg/mL)</th>
<th>Conjugation degree(mg/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>76/4/5/5/10</td>
<td>0.25</td>
<td>$(3.43\pm0.21) \times 10^{-5}$</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>$(3.79\pm0.25) \times 10^{-5}$</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>$(5.46\pm0.47) \times 10^{-5}$</td>
</tr>
<tr>
<td></td>
<td>2.00</td>
<td>$(7.27\pm0.60) \times 10^{-5}$</td>
</tr>
<tr>
<td>71/4/5/10/10</td>
<td>0.25</td>
<td>$(4.54\pm0.45) \times 10^{-5}$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>NIPAAm-DBA-NAS-(NAS-biotin)-HEMA Hydrogel</th>
<th>Anti IL-1β peptide concentration(mg/mL)</th>
<th>Conjugation degree(mg/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>76/4/5/5/10</td>
<td>0.25</td>
<td>$(2.82\pm0.03) \times 10^{-5}$</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>$(3.58\pm0.41) \times 10^{-5}$</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>$(3.79\pm0.43) \times 10^{-5}$</td>
</tr>
<tr>
<td>71/4/5/10/10</td>
<td>0.25</td>
<td>$(3.09\pm0.19) \times 10^{-5}$</td>
</tr>
</tbody>
</table>
Figure 6.1 Synthesis scheme of copolymer NIPAAm-co-DBA-co-NAS-co-HEMA.
Figure 6.2 Synthesis scheme of biotinylation of hydrogel polymer.
Figure 6.3 Scheme of peptide conjugation on the biotinylated hydrogel surface via biotin-avidin interaction.
Figure 6.4 Animal model of limb ischemia and implantation.
Figure 6.5 $^1$H-NMR spectrum of polymer NIPAAm-co-DBA-co-NAS-co-HEMA.
Figure 6.6 $^1$H-NMR spectrum of biotinylated polymer NIPAAm-co-DBA-co-NAS-co-NHS(Biotin)-co-HEMA.
Figure 6.7 Hydrogel solution was flowable at 4°C; Hydrogel solution forms gel at 37°C; Solid hydrogel was flexible.
Figure 6.8 Degradation of hydrogel with different NAS ratio in DPBS at 37°C for 8 week.
Figure 6.9  dsDNA content of NSCs culture in 5%, 10%, and 20% hydrogel at normal culture condition for 7 days. N5: 5% hydrogel, N10: 10% hydrogel, N20: 20% hydrogel.
Figure 6.10 Neural differentiation gene expression of NSCs culture in 5%, 10%, and 20% hydrogel at normal culture condition for 7 days. N5: 5% hydrogel, N10: 10% hydrogel, N20: 20% hydrogel.
Figure 6.11 dsDNA content of NSC/Gel, NSC/Gel0.25ATP, and NSC/Gel1.00ATP at TNF-α culture condition for 7 days. And live cell images of N: NSC/Gel, NLTP: NSC/Gel0.25ATP, NHTP: NSC/Gel1.00ATP. Scale bar: 50μm.
Figure 6.12 Neural differentiation gene expression of NSC/Gel, NSC/Gel0.25ATP, and NSC/Gel1.00ATP at TNF-α culture condition for 7 days. NSC cultured on plate was regarded as control.
Figure 6.13  GFAP, NESIN, and MAP2 protein expression of NSC/Gel, NSC/Gel0.25ATP, and NSC/Gel1.00ATP at TNF-α culture condition for 7 days.
Figure 6.14  dsDNA content of NSC/Gel, NSC/Gel0.25AIP, and NSC/Gel1.00AIP at IL-1β culture condition for 7 days. And live cell images of N: NSC/Gel, NLIP: NSC/Gel0.25AIP, NHIP: NSC/Gel1.00AIP. Scale bar: 50μm.
Figure 6.15 Neural differentiation gene expression of NSC/Gel, NSC/Gel0.25AIP, and NSC/Gel1.00AIP at IL-1β culture condition for 7 days.
Figure 6.16  GFAP, NESTIN, and MAP2 protein expression of NSC/Gel, NSC/Gel0.25AIP, and NSC/Gel1.00AIP at IL-1β culture condition for 7 days.
Figure 6.17 dsDNA content of NSC/Gel, NSC/Gel0.25ATPAIP, and NSC/Gel1.00ATPAIP at TNF-α and IL-1β culture condition for 7 days.
Figure 6.18  Neural differentiation gene expression of NSC/Gel, NSC/Gel0.25ATPAIP, and NSC/Gel1.00ATPAIP at TNF-α and IL-1β culture condition for 7 days.
Figure 6.19 Neural protein expression of NSC/Gel, NSC/Gel0.25ATPAIP, and NSC/Gel1.00ATPAIP at TNF-α and IL-1β culture condition for 7 days.
Figure 6.20  dsDNA content of MSCs culture in 5%, 10%, and 20% hydrogel at normal culture condition for 7 days. M5: 5% hydrogel, M10: 10% hydrogel, M20: 20% hydrogel.
Figure 6.21 Neural differentiation gene expression of MSCs culture in 5%, 10%, and 20% hydrogel at normal culture condition for 7 days. MSC5: 5% hydrogel, MSC10: 10% hydrogel, MSC20: 20% hydrogel. MSC culture in plate was regarded as control.
Figure 6.22 dsDNA content of MSC/Gel, MSC/Gel0.25ATP, MSC/Gel0.50ATP, and MSC/Gel1.00ATP at TNF-α culture condition for 7 days. And live cell images. Scale bar: 50μm.
Figure 6.23  Differentiation gene expression of MSC/Gel, MSC/Gel0.25ATP, MSC/Gel0.50ATP, and MSC/Gel1.00ATP at TNF-α culture condition for 7 days.
Figure 6.24  GFAP protein expression of MSC/Gel, MSC/Gel0.25ATP, MSC/Gel0.50ATP, and MSC/Gel1.00ATP at TNF-\(\alpha\) culture condition for 7 days.
Figure 6.25 NESTIN protein expression of MSC/Gel, MSC/Gel0.25ATP, MSC/Gel0.50ATP, and MSC/Gel1.00ATP at TNF-α culture condition for 7 days.
Figure 6.26 MAP2 protein expression of MSC/Gel, MSC/Gel0.25ATP, MSC/Gel0.50ATP, and MSC/Gel1.00ATP at TNF-α culture condition for 7 days.
Figure 6.27 dsDNA content of MSC/Gel, MSC/Gel0.25AIP, MSC/Gel0.50AIP, and MSC/Gel1.00AIP at IL-1β culture condition for 7 days. And live cell images. Scale bar: 50μm.
Figure 6.28  Differentiation gene expression of MSC/Gel, MSC/Gel0.25AIP, MSC/Gel0.50AIP, and MSC/Gel1.00AIP at IL-1β culture condition for 7 days.
Figure 6.29  GFAP protein expression of MSC/Gel, MSC/Gel0.25AIP, MSC/Gel0.50AIP, and MSC/Gel1.00AIP at IL-1β culture condition for 7 days.
Figure 6.30  NESTIN protein expression of MSC/Gel, MSC/Gel0.25AIP, MSC/Gel0.50AIP, and MSC/Gel1.00AIP at IL-1β culture condition for 7 days.
Figure 6.31  MAP2 protein expression of MSC/Gel, MSC/Gel0.25AIP, MSC/Gel0.50AIP, and MSC/Gel1.00AIP at IL-1β culture condition for 7 days.
Figure 6.32 dsDNA content of MSC/Gel, MSC/Gel0.25ATPAIP, MSC/Gel0.50ATPAIP, and MSC/Gel1.00ATPAIP at TNF-α and IL-1β culture condition for 7 days.
Figure 6.33 Differentiation gene expression of MSC/Gel, MSC/Gel0.25ATPAIP, MSC/Gel0.50ATPAIP, and MSC/Gel1.00ATPAIP at TNF-α and IL-1β culture condition for 7 days.
Figure 6.34  GFAP protein expression of MSC/Gel, MSC/Gel0.25ATPAIP, MSC/Gel0.50ATPAIP, and MSC/Gel1.00ATPAIP at TNF-α and IL-1β culture condition for 7 days.
Figure 6.35 NESTIN protein expression of MSC/Gel, MSC/Gel0.25ATPAIP, MSC/Gel0.50ATPAIP, and MSC/Gel1.00ATPAIP at TNF-α and IL-1β culture condition for 7 days.
Figure 6.36 MAP2 protein expression of MSC/Gel, MSC/Gel0.25ATPAIP, MSC/Gel0.50ATPAIP, and MSC/Gel1.00ATPAIP at TNF-α and IL-1β culture condition for 7 days.
Figure 6.37  *In vivo* live cell images and live cell numbers of MSC/Gel, MSC/GelATP, MSC/GelAIP, and MSC/GelATPAIP ischemic limb implantation for 2 weeks. CG: MSC/Gel, CTG: MSC/GelATP, CIG: MSC/GelAIP, and CTIG: MSC/GelATPAIP.
Figure 6.38 Ki67 and NESTIN staining of MSC/Gel, MSC/GelATP, MSC/GelAIP, and MSC/GelATPAIP ischemic limb implantation for 2 weeks. CG: MSC/Gel, CTG: MSC/GelATP, CIG: MSC/GelAIP, and CTIG: MSC/GelATPAIP.
Figure 6.39  MHC and vWF staining of MSC/Gel, MSC/GelATP, MSC/GelAIP, and MSC/GelATPAIP ischemic limb implantation for 2 weeks. CG: MSC/Gel, CTG: MSC/GelATP, CIG: MSC/GelAIP, and CTIG: MSC/GelATPAIP.
Figure 6.40  Ki67 positive cell numbers of MSC/Gel, MSC/GelATP, MSC/GelAIP, and MSC/GelATPAIP ischemic limb implantation for 2 weeks. CG: MSC/Gel, CTG: MSC/GelATP, CIG: MSC/GelAIP, and CTIG: MSC/GelATPAIP.
Figure 6.41 MHC positive cell numbers of MSC/Gel, MSC/GelATP, MSC/GelAIP, and MSC/GelATPAIP ischemic limb implantation for 2 weeks. CG: MSC/Gel, CTG: MSC/GelATP, CIG: MSC/GelAIP, and CTIG: MSC/GelATPAIP.
Figure 6.42  NESTIN positive cell numbers of MSC/Gel, MSC/GelATP, MSC/GelAIP, and MSC/GelATPAIP ischemic limb implantation for 2 weeks. CG: MSC/Gel, CTG: MSC/GelATP, CIG: MSC/GelAIP, and CTIG: MSC/GelATPAIP.
Figure 6.43  Blood vessel density of MSC/Gel, MSC/GelATP, MSC/GelAIP, and MSC/GelATPAIP ischemic limb implantation for 2 weeks. CG: MSC/Gel, CTG: MSC/GelATP, CIG: MSC/GelAIP, and CTIG: MSC/GelATPAIP.
CHAPTER 7: Conclusion and future work

7.1 Conclusions

The cell survival issues of applying cell therapy to regenerate ischemic induced tissue damage were addressed from several aspects in the work. Series of biodegradable thermosensitive hydrogels were developed to serve as carriers of cells. Pro-survival growth factor, functional oxygen releasing microspheres, and ant-inflammation peptides were introduced to the hydrogel delivery system to improve the survival and differentiation of the cells.

In Chapter 2, an injectable thermosensitive biodegradable hydrogel based bFGF release system was developed to improve stem cell survival and angiogenesis under ischemic conditions. The system was readily injectable and capable of releasing bioactive bFGF for 4 weeks. In vitro results showed the survival of CDCs could be successfully enhanced when they were encapsulated in bFGF releasing hydrogel under ischemic conditions. In vivo results indicated that cell survival, engraftment with host tissues and proliferation were significantly improved when delivering CDCs in bFGF releasing hydrogel. In addition, angiogenesis and cell differentiation were promoted in the bFGF releasing hydrogel injected group.

In Chapter 3, an injectable hydrogel based oxygen release system was developed to improve cell survival under ischemic conditions in vitro and in vivo. The hydrogel was
thermosensitive and biodegradable. The system was injectable and capable of sustained releasing oxygen at certain level to support cell consumption for at least 2 weeks. CDCs and NSCs were encapsulated in the developed hydrogel based oxygen releasing system and the survival of both cells was enhanced under ischemic conditions. The survived NSCs demonstrated the ability to differentiate into neural cells at gene and protein level. Paracrine effect of the survived CDCs was detected under ischemic conditions. The survival and proliferation of the stem cells resulted from the released oxygen and stimulated paracrine effect which helped the cells overcome the harsh ischemic environment by supplying cells with oxygen and activation of proliferation pathway. In addition, CDCs which were implanted into rat MI heart with oxygen releasing system could proliferation and differentiation in cardiac tissue.

In Chapter 4, an imagable oxygen releasing system was successfully developed. The oxygen releasing function was based on core-shell structured microspheres fabricated in Chapter 3 and fluorescent agent HYP. The newly developed imagable oxygen releasing system was able to release oxygen for at least 28 days to promote cell survival under ischemic conditions while fluorescent imagable in vitro and in vivo. This imagable system provided a promising and beneficial way in clinical trials in helping monitoring the conditions of oxygen releasing microspheres in vivo by simply visualizing the injected oxygen releasing system in the machine without any invasion.

In Chapter 5, a hypoxia-sensitive organic compound (NI) was synthesized and applied to modify a designed hydrophobic biodegradable polymer. The hydrophilic and hydrophobic properties of the organic compound NI modified polymer were proved to be
sensitive to the oxygen content from the significant different between their water content and water contact angle in the environment. When the oxygen level changed from normal to hypoxic, the NI modified polymer converted to be hydrophilic via bioreduction process. Hypoxia-sensitive oxygen releasing microspheres were fabricated with the material. Releasing kinetics of the microspheres was related to the amount of NI in the shell material. The fabricated oxygen releasing microspheres with shell polymer at a high NI ratio performed quick and high oxygen release. Further, catalase was also successfully conjugated onto the shell surface of the microspheres and the release of oxygen from the microspheres was proved to be similar as usual. This hypoxia-sensitive oxygen releasing microspheres were able to enhance cell survival under ischemic conditions as well as stimulate paracrine effect of the survived cells. The quick release of oxygen of hypoxia – sensitive under ischemic conditions should benefit large oxygen demand in the very beginning of the ischemic tissue regeneration.

In Chapter 6, we developed an immunoprotective hydrogel system to successfully prevent the penetration of pro-inflammatory cytokines and therefore enhanced the encapsulated cells survival under harsh environment with immune rejection in vitro and in vivo. The fabricated immunoprotective hydrogel was conjugated with anti-inflammatory peptides on the surface which demonstrated high binding affinity to the pro-inflammatory cytokines TNF-α and IL-1β. The protective effect of the system related with the conjugation degree of the peptides on the hydrogel. The survival rate of the encapsulated stem cells was significantly increased by conjugating both peptides as well as the oxygen releasing microspheres in vitro and in vivo. The encapsulated NSCs and MSCs also showed
neural differentiation properties in the hydrogel of selected concentration and the differentiation was promoted in the anti-inflammatory peptides conjugated hydrogel system \textit{in vitro} and \textit{in vivo}.

\section*{7.2 Future work}

In this work, stem cell survival under ischemic conditions and inflammation conditions were improved by thermosensitive hydrogel based cell delivery system. Large amounts of \textit{in vitro} cell experiments were conducted to evaluate the functions of the developed cell delivery systems. To ensure the method was therapeutically applicable, more animal experiments are required.

To illustrate the efficiency of using bFGF releasing system and oxygen releasing system to regenerate MI heart, cardiac protection of the mechanical support of the hydrogel to the cardiac wall, pro survival growth factor, and supplied oxygen to prevent further tissue damage should be evaluated. Pathology study of the heart should be performed to investigate the inflammation degree in the infarct area and the wall thickness of left ventricle. Heart function tested by echocardiogram was an option to characterize the function restoration after the surgery.

Peptide binding affinity to the cytokines should be measured to support the efficiency of cytokine blockage of the developed anti inflammation hydrogel. As the gel degrades, protection of the hydrogel in a relatively long time under inflammation conditions should be tested to illustrate the utility time of the system. The more cell types can be encapsulated...
into the hydrogel system and implant into different animal models with inflammation conditions to study the protection of the system.

To make the imagable oxygen releasing system a solid detection approach, interaction of the fluorescent agent HYP with other components in the complex should be studied to determine an upper and lower level of HYP encapsulation capability and detection limitations. *In vivo* fluorescent imaging experiments should perform on several animal models to test the versatility of the system and at the same time accumulated experiences with the new imaging system would guide further therapeutic trials.

The oxygen releasing kinetics significantly depended on the properties of the shell materials and the environment. Degradable polymer with different molecular weight and the size of fabricated microspheres resulted in different releasing behavior. Seeking ways to synthesize polymer with specific range of molecular weight and optimize the electrospray conditions to produce oxygen releasing microspheres with consistent size and shell thickness should be a fundamental but important works. How the hypoxia-sensitive material changes the releasing behavior should be further investigated by using microspheres with a series NI amount and the release should be conducted under several different environments with different oxygen level.


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