INJECTABLE ALGINATE HYDROGELS WITH GROWTH FACTORS / LIVING STEM CELLS FOR MYOCARDIAL INFARCTION REPAIR

A Thesis Submitted to the

College of Graduate and Postdoctoral Studies

in Partial Fulfillment of the Requirements

for the degree of Master of Science

in the Division of Biomedical Engineering

University of Saskatchewan

Saskatoon

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Abstract

Myocardial infarction (MI), caused by the occlusion of the left ventricular coronary artery, may lead to massive loss of cardiomyocytes and eventually heart failure. To repair MI, one key issue is to compensate for the loss of cardiomyocytes in the MI site. Notably, cardiomyocytes are mature cells with limited proliferation capability. Another issue in MI repair is to improve the local ischemic condition at the MI site so as to provide the cells with nutrition and oxygen, where the revascularization or the renewal of the small vessels (or angiogenesis) is critical. To address the issues, the proposed study aims to develop injectable alginate hydrogels with growth factor/living cells for MI repair. To be more specific, two specific strategies were developed, which were 1) to co-deliver 6-Bromoindirubin-3-oxime (BIO) and insulin-like growth factor (IGF-1) by means of nanoparticles and 2) to encapsulate living stem cells. BIO is a small molecular drug that can promote the regeneration of cardiomyocytes and IGF-1 is able to stimulate the angiogenesis.

In the development of strategy 1), both BIO and IGF-1 were encapsulated in gelatin nanoparticles, which were later cross-linked with the oxidized alginate to form a novel hybrid hydrogel system. The fabricated nanoparticles were then subjected both *in vitro* and *in vivo* characterization. The results have shown that the growth factors can continue to release over 110 hours *in vitro* and last for 45 days *in vivo*. The *in vivo* results have also shown that the hybrid system could enhance the proliferation of cardiomyocytes *in situ* and could promote revascularization around the MI sites, allowing improved cardiac function. Taken together, the strategy of co-delivering of BIO and IGF-1 is promising for MI repair.

In the development of strategy 2), two types of alginate hydrogels, based on self-crosslinking (SCL) and calcium ion crosslinking (Ca^{2+}), were synthesized in varying formulations; hydrogels encapsulated living muscle-derived stem cells (MDSCs) and their performance was evaluated in terms of optimizing cell viability during the injection process as well as the live/dead ratio after long-term cultivation. The morphology of the hydrogel-encapsulated cells was characterized by scanning electronic microscopy (SEM) and live/dead cells were examined using an 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide staining (MTT) assay. The mechanical properties of the hydrogels were also determined via a rheometer, to identify their influence on cell viability during the injection process and with respect to long-term cultivation. The results show

that living cells are able to survive in both types of hydrogels and SCL hydrogel in particular is better for the living cells in long term.

To sum up, this study illustrates that both strategies are promising in MI repair by presenting a non-invasive method of injecting biocompatible and biodegradable hydrogel with growth factors and living cells for supplementing cells and angiogenesis for further enhancing heart function in MI rats. These strategies could be adopted and used in clinical applications to human patients.

Acknowledgements

This thesis is dedicated to my family.

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List of Abbreviations

MI	myocardial infarction
ECM	extracellular matrix
MMP	matrix metalloproteinases
3D	three dimension
CICL	calcium-ion-cross-linked
ADA	Alginate dialdehyde
SCL	self-cross-linked
VEGF	vascular endothelial growth factor
PCR	polymerase chain reaction
BIO	6-Bromoindirubin-3-oxime
IGF-1	insulin-like growth factor
DMEM	Dulbecco's modified Eagle medium
MDSC	muscle derived stem cells
NPs	nanoparticles
BgNPs	Blank gelatin NPs
OgNPs	BIO-loaded gelatin NPs
FgNPs	IGF-1-loaded gelatin NPs
CgNPs	BIO-IGF1 co-loaded gelatin NPs
DMSO	dimethyl sulfoxide
SD	Sprague–Dawley
UV	ultraviolet
SEM	scanning electron microscope
E%	entrapment efficiency
FBS	fetal bovine serum
LV	left ventricular
LVEF	left ventricular ejection fraction
FS	fractional shortening

LVSD   left ventricular end systolic diameter

LVDD   left ventricular end diastolic diameter

2D   two dimension

PCNA   proliferating cell nuclear antigen

PDI   polydispersity index

H&E   Hematoxylin and Eosin

MTT   3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Ca^{2+} calcium ion crosslinking

bFGF   basic fibroblast growth factor

PDGF   platelet derived growth factor

$MgCl_2$ Magnesium chloride

CIHR   Canadian Institutes of Health Research

1 Introduction and Objectives

1.1 Background of Myocardial Infarction

Myocardial infarction (MI), commonly known as heart attack, results from interruption of the blood supply to a part of the heart. This is most commonly due to occlusion of the coronary artery. If left untreated, the ischemia or resulting restriction in blood supply may cause damage or death of the myocardium or cardiac muscle tissue, thus leading to heart failure. According to the latest report from the World Heath Organization [1], MI and other cardiovascular diseases are the leading causes of death worldwide.

Hearts are primarily composed of the myocardium and coronary arteries. The myocardium has extremely limited regeneration potential [2, 3]. Supplied with oxygen- and nutrient-rich blood through the coronary arteries, the myocardium continuously contracts and relaxes so as to pump blood throughout the body. After MI, the ischemic myocardium ceases to function properly due to the loss or death of cardiomyocytes, i.e., the cells of which the myocardium is comprised. This is further accompanied by a ventricular remodelling process [4, 5] due to the degradation of the extracellular matrix (ECM) by matrix metalloproteinases (MMP) [6, 7] and scar tissue formation [8], eventually leading to congestive heart failure [9].

Various therapies, including pharmaceutical interventions, have been exploited to slow down the progression to heart failure. Unfortunately, these therapies do not restore function to the affected myocardium [9, 10]. Heart transplantation is the gold standard to treat congestive heart failure but is severely limited as an option due to the limited availability of donor organs. Moreover, the immunological injection response in the hosts after the heart transplantation surgery is a potential risk [11, 12] and a crucial factor lowering life quality of patients, which makes this therapy less practical clinically.

With advances in tissue engineering, injection or implantation of tissue engineering cardiac

constructs or scaffolds made from biomaterials and/or living cells into the affected myocardium shows promise for improving MI repair.

1.2 Literature Review on Injection Strategies

1.2.1 Cardiomyoplasty and Cell Sources

A therapy is demonstrated through recent investigations into cellular cardiomyoplasty, wherein cardiomyoblasts or other types of stem cells are directly injected into the injured heart, serving as renewable cellular sources aiding in the repair of the damaged myocardium [3]. Studies working on improvement in myocardial function by means of cardiomyoplasty have been demonstrated [3, 13, 14, 15].

Cardiomyocytes are mature mammal cells that are limited in regeneration [16, 17]. By transplanting exogenous cells into the damaged heart after MI, it is an important way to supplement the cells for repairing MI [18]. Various stem cells show the ability to differentiate into cardiomyocytes and proliferate as candidates of cells sources [19, 20].

Embryonic stem cells transplantation

Research has illustrated that embryonic stem cells can form new myocardium after transplanting into normal or infarcted heart [21], and that stem cell derived cardiomyocytes can improve the myocardial performance in MI rat models [22]. Also, due to the facts that embryonic stem cells can from an efficient alternative substrate in MI rats hearts, the advantages for using these cells in MI rats are as of preventing myocardial wall from thinning and improving the contractility [23]. As such, embryonic stem cells are considered as an appropriate cell type for supplementing cell loss in MI heart. However, it is also noted by experiments that human stem cells cannot directly form new myocardium after transplantation into MI rat hearts and may create teratoma. However, they can attenuate post-MI scar thinning and left ventricular dysfunction [24]. Another previous study indicated another disadvantage of embryonic stem cells in MI treatment is causing tumour genesis [25]. Moreover, the source of embryonic stem cells is limited because these cells are still not considered ethical for use in clinical trials.

Bone marrow stem cells

Bone marrow stem cells retain the regenerative ability of forming new cardiomyocytes and coronary vessels in MI rat models [26]. Moreover, they have more sources compared with embryonic stem cells, which solves the deficiency of limited access of embryonic stem cells. Zhang et al. advocate these aforementioned conclusions by illustrating the experimental results that nine days after transplantation of bone marrow stem cells in MI mice, the new generated myocardium occupies 68% in the infarcted area [27]. Tanaka et al. state that heart function was improved, angiogenesis was enhanced [28] and the regenerative ability for deriving into cardiomyocytes was improved after transplanting bone marrow mesenchyme stem cell into MI rats [29]. However, disadvantages of bone marrow stem cells, such as difficult accessibility and disease-related malfunction, discussed in these studies [30] [31] [32] have been noted.

Adipose derived stem cells

Adipose derived stem cells are promising candidates for repairing MI due to their high potential ability for deriving into cardiomyocyte-like cells and improving heart function after being transplanted into MI rat' hearts [33]. Adipose derived stem cells can be obtained from adipose tissue, which guarantees the abundant resource [34]. This is proved by Choi et al.'s experimental results that have shown modified heart function and enhanced vascularization as well as improved long-term survival rate after transplanting adipose derived stem cells into acute MI rats [35, 36, 37]. On the other hand, some research point out that mesenchymal stem cells from adipose tissue cannot differentiate into real cardiomyocytes although the heart function is improved after being transplanted [38].

Induced pluripotent stem cells

Induced pluripotent stem cells, another type of cells of high differentiation and proliferation ability with a plentiful of access [39], may also be an appropriate cell type in MI repair. Experimental results show that induced pluripotent stem cells maintain cell viability by inhibiting apoptosis via Akt/Pten pathway in doxorubicin-induced cardiotoxicity [40]. Facts showing induced pluripotent stem cells are beneficial for heart function repair after MI, such as in MI mice models the cardiac

remodeling can be attenuated by the absence of induced pluripotent stem cells derived cardiomy-ocyte sheets [41]. Jia et al. illustrate that heart function is improved by intramyocardial injection of induced pluripotent stem cells in acute MI porcine models [42]. Similarly, heart repair and left ventricular dysfunction can be ameliorated in acute and subacute MI rats respectively by intra-myocardial delivery of induced pluripotent stem cells [43, 44]. However, shortages of induced pluripotent stem cells are emerging and make it difficult when applying in MI repair. For example, the reprogramming of induced pluripotent stem cells is still slow and inefficient [45], which costs more time and energy to take usage of these cells than other cells. Another disadvantage is that the immunological rejection responses are serious after transplanting induced pluripotent stem cells [46].

Muscle derived stem cells

Muscle derived stem cells, owing to their characteristics of regeneration and great differentiation potential into cardiomyocytes, are a kind of somatic stem cells which have plentiful of sources [47, 48]. Moreover, myosphere-derived progenitor cells have an extended period of proliferation time. They can spontaneously differentiate into myotubes in differentiation media and into other meso-dermal cell lineages in induction media [49]. Several previous studies reported that muscle derived stem cells have good proliferative and adhesion properties. Muscle derived stem cells can improve cell regeneration capacity and pumping ability of heart in chronic MI model without causing any significant arrhythmias. Currently no obvious shortages have been reported of the application of muscle-derived stem cells in MI repair. They are considered appropriate cell source type, though the retention rate is still too low after transplanting into MI hearts.

1.2.2   Injectable Hydrogels

The cellular therapy faces a major problem. Typically, the cell injection process transplants cells in an aqueous solution and, as such, does not provide cells with any environment or matrix for attachment and protection. In such harsh conditions, the transplanted cells may be damaged [50, 51]. Furthermore, upon injection into the ischemic myocardium, cells experience a harsh hypoxic environment that further negatively impacts cell viability [52, 53, 54, 55, 56]. As a result, most (90-95%) injected cells die shortly after transplantation and only a small subpopulation plays a role in

muscle regeneration [55, 56]. More recently, research has shown cell viability during injection can be significantly improved if cells are encapsulated in hydrogels rather than suspended in aqueous solutions [57, 56, 58, 59].

Hydrogels are cross-linked networks of polymers, such as fibrin and alginate, with high water content between polymer chains [60, 61, 62, 63]. When used for cell encapsulation, hydrogels provide a hydrated tissue-like scaffold or environment for cell attachment and protection, thereby improving cell viability. Among various injectable hydrogels, alginate cross-linked in the presence of calcium ions has been drawing considerable attention for use in MI repair, leading to enhanced cell viability and proliferation as well as improved contractile function [64, 65, 66]. However, the calcium-ion-cross-linked (CICL) alginate hydrogel has uncontrollable degradation kinetics [67, 68] and poor cell adhesion and infiltration [61]. Recently, successful application of a new class of hydrogels from oxidized alginate and gelatin, through a self-cross-linked (SCL) process, has been reported for drug delivery and wound healing [64, 65]. Our group have begun to synthesize and characterize SCL alginate hydrogels, and demonstrated them to be more suitable for MI repair than CICL alginate hydrogels. One key objective of this MSc project is to expand our ongoing studies to synthesize SCL alginate hydrogels for cell encapsulation in MI repair.

1.2.3   Controlled Release Strategies in MI Repair

Drugs or growth factors may be used in MI repair to stimulate cardiomyocytes to proliferate. For this, drugs should be small molecule and should permeate into cells, meanwhile not cause toxic effect. The challenge here is to control drug release rate or speed such that the drug concentration can be maintained constant for the MI repair.

One feasible way to control the release speed is entrapping drugs by nanoparticles for gradually releasing from particles [66, 69]. Another way for controlling the release speed is to cross-link nanoparticles with scaffolds via chemical, combining or disconnecting chemical bonds [70, 71]. Hua et al. released diclofenac sodium through the drug delivery system of cross-linking Ca^{2+} with sodium alginate/poly (vinyl alcohol) by adjusting the pH value for heart function recovery examination [72].

Controlled release can be applied in a broad manner, such as cell release with particular drugs. Kasko et al. discussed repairing heart function via releasing living cells and drugs together, par-

ticularly patterned hydrogel has been fabricated based on two-photon lithography technique for releasing cells in local MI area [73]. Due to plentiful applications of controlled release of nanoparticles crosslinking with hydrogel, it is critical to manipulate the loading efficiency of nanoparticles. Hamidi et al. proposed a new drug delivery model of valproate-loaded nanogels encapsulated erythrocytes showed reasonable entrapment efficiency of 42.07 $\pm$ 3.6% of drug loading [74]. Lapointe et al. indicate that thermoresponsive materials such as poly (N-isopropylacrylamide) based nanoparticles could be embedded in hydrogel and have a better application of drug delivery than traditional method [75]. Chen et al studied in vitro skin permeation experiments for the measurement of efficiency of drug-loaded nanoparticles delivery, particularly these nanoparticles having average diameters of about 30 nm and 100 nm and their structure is composed of inner spHerical solid spheres and an outer hydrogel matrix [76].

A breakthrough method for local drug release is fabricating *in situ* cross-link hybrid hydrogel composed of polysaccharides (hyaluronic acid, dextran and/or carboxymethyl cellulose) and gelatin, showing the ability for releasing proteins with minimal cytotoxicity [77]. Another case is *in situ* thermoreversible gel comprising liposome-containing paclitaxel is studied as controlled release method and can improve the antitumor drug efficiency [78].

1.3   Research Objectives

From the above literature review, it is known that the challenge for MI repair with tissue engineering strategies is to supplement functional cells to the MI site, meanwhile keeping the cells alive and functioning. To overcome this challenge, this thesis aims to develop injectable alginate hydrogels for the controlled release of growth factors and the encapsulation of living cells. Two main specific research objectives are set and outlined as below:

1.3.1   Co-delivery of BIO and IGF-1 in Nanoparticles by Hydrogel

Differentiation and proliferation of endogenous cardiomyocytes *in situ* can effectively improve cardiac repair following myocardial infarction (MI). This objective is to perform a study on the sustained co-delivery of BIO and IGF-1 in a hybrid hydrogel system to simulate endogenous cardiac repair in an MI rat model. For this, both BIO and IGF-1 are to be encapsulated in gelatin nanoparti-

cles, which are later cross-linked with the oxidized alginate to form a novel hybrid hydrogel system. Then, *in vitro* and *in vivo* tests are to be performed so as to illustrate the effectiveness of the hybrid hydrogel system for co-delivery BIO and IGF-1 to the MI area, in terms of the proliferation of cardiomyocytes and revascularization.

1.3.2   Synthesize Alginate Hydrogels and Optimize Cell Viability

High cell retention rate, cell viability and the long term cell live/dead ratio after transplanting living cells are important in MI repair. This is needed to supplement the functional cell in MI area as an effective way for enhancing heart function. This objective is to fabricate injectable hydrogels encapsulating living cells, which is easier to inject cells into the MI area with fewer trauma during the surgery, then test and optimize the cells viability after injection by selecting the components of the hydrogels. For this, the injectable alginate hydrogel of different compositions with two methods briefly described as self-crosslinking hydrogel and cross-linker (Ca^{2+}) adjusted hydrogel are to be synthesized. The living stem cells are to be encapsulated into both types of hydrogels and the cells viability and long-term live/dead ratio are to be examined.

1.4   Thesis Organization

This thesis is composed of four chapters. Chapter 1 gives the introduction and research objectives of this thesis; Chapters 2 and 3 present the work related to the two research objectives; Chapter 4 discusses the conclusions drawn from this thesis as well as the future work.

Chapter 1 briefly introduces the background of myocardial infarction and presents the literature review on tissue engineering treatments for MI repair; and on this basis, this chapter defines two research objectives to be achieved.

In chapter 2, the first research objective and methods as well as results of the controlled release of BIO and IGF-1 for MI repair in animal models have been described and discussed.

Briefly, a pH sensitive hybrid hydrogel system combining with nanoparticles for co-delivery of BIO and IGF-1 for simulating endogenous cardiac repair in MI rat models was fabricated. Growth factors BIO and IGF-1 were aimed to be efficiently encapsulated into gelatin nanoparticles, and then these nanoparticles were cross-linked with the oxidized alginate hydrogel for forming a novel

hybrid hydrogel system. With the change of pH value in the micro-envrionment after MI in response of time growing, both growth factors were released for the specific concentration via disconnecting from the hydrogel. The release profile of the nanoparticles from the hydrogel and the release of the small molecular growth factors from nanoparticles were described. Taken together all *in vivo* results, it indicates that this hybrid hydrogel system have enhanced the proliferation of cardiomyocytes and promoted the angiogenesis in the MI area, which further improved the cardiac function.

In chapter 3, the research objectives and methods as well as experimental results of encapsulating living stem cells in alginate hydrogel for MI repair have been described and discussed.

Generally, two types of injectable alginate hydrogels based on self-crosslinking (SCL) and calcium ion crosslinking (Ca^{2+}) with various components were synthesized. Then living muscle derived stem cells were encapsulated into both types of hydrogels, for their performance evaluation in terms of optimizing cells viability during the injection process, and long term cells live/dead ratio after two months cultivation. The characters of both types of hydrogels and the behaviour of living cells were studied. By comparing the cells viability and long term cells live/dead ratio in both types of hydrogels in varying formulations, the appropriate composition of hydrogel was selected. Overall, the SCL hydrogel with a 0.8% alginate and 20% gelatin has the highest cells viability during the injection process and the Ca^{2+} hydrogel with a 1.1% alginate and 20% gelatin has the highest cells live/dead ratio after two months of cultivation.

In chapter 4, the conclusions based on the research objectives and obtained results were summarized and future work has been described.

2 Sustained Co-delivery of BIO and IGF-1 by a Novel Hybrid Hydrogel System to Stimulate Endogenous Cardiac Repair in Myocardial Infarcted Rat Hearts

Author contributions

Rui Fang performed the majority of the research and wrote the paper. Shupei Qiao helped revise the paper. Yi Liu, Qingyuan Meng, Bing Song and Xiaolu Hou helped with *in vivo* tests and measurements. Weiming Tian and Xiongbiao Chen supervised the research and revised the paper.

2.1 Abstract

Dedifferentiation and proliferation of endogenous cardiomyocytes *in situ* can effectively improve cardiac repair following myocardial infarction (MI). 6-Bromoindirubin-3-oxim (BIO) and insulin-like growth factor (IGF)-1 are two potent factors that promote cardiomyocyte survival and proliferation. However, their deliveries for sustained release in MI-affected areas was proven to be challenging. In the current paper, we present a study on the sustained co-delivery of BIO and IGF-1 in a hybrid hydrogel system to simulate endogenous cardiac repair in a MI rat model. Both BIO and IGF-1 were efficiently encapsulated in gelatin nanoparticles, which were later cross-linked

with the oxidized alginate to form a novel hybrid hydrogel system. The *in vivo* results indicated that the hybrid system could enhance the proliferation of cardiomyocytes *in situ* and could promote revascularization around the MI sites, allowing improved cardiac function. Taken together, we concluded that the hybrid hydrogel system can co-deliver BIO and IGF-1 to areas of MI and improve cardiac function by promoting the proliferation of cardiomyocytes and revascularization.

2.2   Introduction

Many heart diseases primarily result in the loss of cardiomyocytes. It has been a significant challenge to develop effective treatments for cardiac repair because adult mammalian cardiomyocytes are highly differentiated cells and have long been thought to undergo terminal differentiation [79]. Contrary to this long-held view, an emerging strategy posits that in response to heart injury, resident cardiomyocytes and cardiac stem cells surrounding the injured area can migrate and rapidly re-enter the cell cycle, thus promoting heart function recovery [80, 81]. However, the endogenous regenerative capacity of hearts is limited due to the massive loss of cardiomyocytes after myocardial infarction (MI) and heart failure. MI is considered a major cardiovascular disease, and it was found to be a major factor that contributed to non-natural mortality worldwide in 2013 [82, 83]. Current stem cell-based therapies have the potential to fundamentally improve the treatments of ischemic cardiac injury and heart failure. These therapeutic approaches mainly involve the use of bone marrow-derived mononuclear cells and their subsets, such as mesenchymal stem/stromal cells, endothelial progenitor cells, as well as adipose tissue-derived muscle stem cells, cardiac tissue-derived stem cells, and cell combinations [84, 85, 86, 87]. However, these stem cell therapies have low efficacy due to poor cell engraftment and differentiation under the harsh (low nutrient and low oxygen) ischemic environment of infarcted hearts [84, 85]. Previous studies have shown that fewer than 2% of cells survive a few weeks after the stem cells' delivery [86, 87, 88]. Despite the availability of many treatment options, heart disease remains the leading cause of death worldwide, raising the great need for novel or innovative therapeutic strategies. A recent study on stimulating cardiomyocyte dedifferentiation and proliferation by activating the mitotic signaling pathways involved in embryonic heart growth represents a complementary approach for heart regeneration and repair [89, 90, 91, 92]. An inhibitor of glycogen synthase kinase-3,6-Bromoindirubin-3-oxim

(BIO), which is isolated from mollusk Tyrian purple indirubins, has been shown able to induce the dedifferentiation of cardiomyocytes or endothelial cells, thus promoting mature cardiomyocyte proliferation [93, 94]. On the other hand, substantial data also illustrate that insulin-like growth factor 1 (IGF-1) is a potent cardiomyocyte growth and survival factor. It has been illustrated that IGF-1 deletion has harmful effects on cardiac growth and its deficiency is associated with an increased risk of cardiovascular disease [95, 96]. Cardiac-specific overexpression of IGF-1 can protect against myocyte apoptosis and ventricular dilation following MI [97, 98]. Recently, drug delivery systems using biomaterials as vehicles have drawn considerable attention. Studies have shown that by combining growth factors with an injectable biomaterial, the biomaterial could serve as a controlled drug-release platform to improve functional outcomes [99, 100]. However, co-delivery of BIO and IGF-1 to the injured heart area remains a challenge. Daily injections of both BIO and IGF-1 is a straightforward method that can maintain these agents at appropriate levels; however, this method causes issues, such as toxicity, due to their high doses [101]. In the current research, we report a novel hybrid hydrogel system of chemically encapsulated gelatin nanoparticles (NPs) for the sustained co-delivery of BIO and IGF-1 in MI treatment.

2.3 Materials and Methods

2.3.1 Materials

Alginate (low viscosity), gelatin, sodium periodate, and hydrochloric acid were purchased from Sigma Aldrich Co. (St. Louis, MO, USA); BIO was purchased from EMD Millipore, a division of Merck KGaA (Darmstadt, Germany); IGF-1 was supplied by ProSpec-Tany TechnoGene Ltd (Rehovot, Israel); and Cy7 (molecular formula: $C_{35}H_{41}KN_2O_8S_2$) was purchased from Fanbo Biochemicals Co (Beijing, People's Republic of China).

Sprague-Dawley (SD) male rats, specific pathogen free (SPF) (weighting 200-250g each), provided by the Laboratory Animal Center of Harbin Medical University (Harbin, People's Republic of China), were used to isolate cardiomyocytes, and served as MI animal models. All animals were fed ad libitum and kept under the normal 12-hour dark/12-hour light cycle. All procedures were approved by the University Ethics Committee of the Harbin Institute of Technology.

2.3.2 Methods

Preparation NPs

Blank gelatin NPs (BgNPs) were prepared using a two-step desolvation method with slight modifications [102, 103]. Briefly, gelatin was dissolved at 5 wt% in distilled water by stirring at $55\,°C$ for 2 hours. Then, the pH of the gelatin solution was adjusted to proceed with the second desolvation step. To prepare the BIO-loaded gelatin NPs (OgNPs), IGF-1-loaded gelatin NPs (FgNPs), and BIO-IGF1 co-loaded gelatin NPs (CgNPs), $10\,\mu L$ of $5\,\mu M$ BIO (dissolved in $500\,\mu L$ of dimethyl sulfoxide [DMSO]) and $10\,\mu L$ of $10\,\mu M$ IGF-1 were added individually or together into 10 mL of 5% gelatin solution in a dark environment respectively, followed by the dropwise addition of acetone ($30\,\mu L$) to form NPs. At the end of the process, a glutaraldehyde solution (25% v/v aqueous solution) was added as a cross-linking agent and the solution was stirred for 12 hours at a speed of 600 rpm. DMSO was removed by means of distilled water. Eventually, the solution was centrifuged at 12,000 rpm for 1.5 hours, yielding the NPs. Dynamic light scattering was conducted using a Zetasizer Nano ZS system (Malvern Instruments, Malvern, UK) to characterize the distribution and size of the NPs.

Encapsulation Efficiencies of NPs

The encapsulation efficiency of the BIO and IGF-1 within the NPs was evaluated using an ultraviolet (UV) method. A drug-encapsulating gelatin NP solution was filtered through a Millipore filter (UFP2THK24 [100 kD cutoff]), where the free drug present in the aqueous buffer passed through the filter, leaving the drug entrapped in the NPs. Following the separation of the NPs from the aqueous buffer, the drug left in the aqueous buffer was measured using a 752 UV/visible recording spectrophotometer with a wavelength of $500\,nm$ and $280\,nm$ respectively. The total amount of drug left in the aqueous solution was subtracted from the amount of drug originally added in the reaction medium, and the entrapment efficiency (E %) was calculated from the ratio of the amount of drug entrapped over the total amount of drug added.

Synthesis of Self-cross-linked Hydrogel and Its Physicochemical Characterization

 Hydrogel was synthesized as previously described [104]. Briefly, oxidized alginate was obtained by mixing sodium peroxide and sodium alginate (in distilled water) with mass ratios of 1:2. The reaction was conducted at $4\,°C$ overnight and the degree of oxidation was evaluated by measuring the concentration of sodium peroxide. Sodium peroxide was left unconsumed after 24 hours and the concentration was measured by titration of hydrochloric acid (0.5 wt%). The oxidized alginate and gelatin hydrogel were then obtained by mixing the alginate dialdehyde and gelatin NP solutions. The gelation process and the mechanical properties of oxidized alginate and gelatin hydrogel were evaluated by examining the time of gelation onset and the evolution of elasticity at $37\,°C$ in the constant strain mode by means of a Bohlin Gemini II rheometer (Malvern Instruments) featuring a parallel plate geometry (40 mm in diameter), as in our previous study [104].

Drug-releasing Profile *in vitro* and *in vivo*

To monitor the *in vitro* release profile, gelatin NPs and each of the encapsulating drugs were made to conform by means of a semi-permeable membrane (40 KD) and were then immersed in $10\,mL$ of phosphate buffered saline at $4\,°C$ for release within a time period of 7 days; every 24 hours, the optical density was examined by a UV spectrophotometer at $500\,nm$. A sample of $1\,cm^3$ of the encapsulating drug (10 uL) was examined under the same conditions as the control group, so as to study the drug's release profile. To monitor the *in vivo* release profile, an *in vivo* imaging method was used. First, the gelatin NPs were labeled with the fluorescent agent Cy7, and then the labeled gelatin NPs were conjugated to the hydrogel, as described in section 2.3.2. Both the labeled gelatin NPs alone, as well as the labeled gelatin NPs that conjugated to the hydrogels, were injected into the area affected by MI in 20 rats (10 rats in each group), as described in a previous study [104]. The fluorescence of both groups was examined and recorded by an *in vivo* imaging system (DXS 4000 PRO; Eastman Kodak Co, Rochester, NY, USA) every 5 days.

Cardiomyocyte Culture

Neonatal cardiomyocytes were isolated from 2- to 3-day-old SD rats, as adapted from a previous protocol [105]. Hearts were removed from the neonatal rats; then, atrial and connective tissues

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were excised. Hearts were minced into $1\,\mathrm{mm}^3$ pieces and then subjected to five rounds of enzymatic digestion for 8–10 minutes with collagenase II ($0.5\,\mathrm{mg\,mL}^{-1}$; Worthington Biochemical Corporation, Lakewood, NJ, USA). Digestion was stopped with an equal volume of Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA, USA). The cell suspension was centrifuged for 5 minutes at 2,000 xg and the resulting pellet was resuspended in DMEM containing 10% FBS. Cells were pre-plated in cell culture flasks to reduce non-myocyte contamination (Corning Incorporated, Corning, NY, USA) for 90 minutes. Isolated cardiomyocytes were made into a suspension using oxidized alginate conjugated with gelatin NPs at a density of 10^5 cells/mL. The obtained cells suspension are a mixture of cardiomyocytes and fibroblasts. Since cardiomyocytes could be stained by anti-sarcomeric antibody via immunofluorescence, the purity of the cardiomyocyte suspension can be evaluated by counting the numbers of anti-sarcomeric antibody positive cells or by visual inspection.

Cell Proliferation in Hydrogels Assay

After 7 days of culture, the hydrogel/cardiomyocyte constructs were fixed. Immunofluorescence was performed with the proliferating cell nuclear antigen (PCNA) antibody (1:200; Abcam plc, Cambridge, UK) to identify the proliferation of cardiomyocytes. Laser confocal images were observed and the PCNA-positive cells were counted.

MI Animal Model Preparation and Hydrogel Implantation

Ninety-five male SD rats (weighting $250\,\mathrm{g}$ each) were operated on to create the MI animal models. The rats were anaesthetized with an intravenous injection of 3% pentobarbital sodium ($30\,\mathrm{mg\,kg}^{-1}$) [106], and an MI was subsequently induced ligating the left anterior descending coronary artery $2\,\mathrm{mm}$ from the top of the normally-positioned left auricle. Rats could be died because of surgery and severe induced MI. We waited for 7 days to make sure there are no rats died because of the above reasons. Otherwise, we have to occlude there rats from the final experimental analysis. One week later, each rat was injected with $80\,\mu\mathrm{L}$ of a given hydrogel (Table 2.1) into the anterior and lateral regions bordering the infarct, as well as at the center of the infarct (16 uL per region). Anaesthetic operation with the same dosage was performed on the rats before the ultrasound evaluation and hydrogel injection.

Table 2.1: Groups and number of animals used for *in vivo* MI repair. Abbreviations: MI, myocardial infarction; BIO, 6-bromoindirubin-3-oxime; IgF-1, insulin-like growth factor 1. Sham animals accidentally died because of the surgery.

Groups	# of animals per group	# of animals survived at 6 weeks post-surgery	Survival rate
Sham	15	12	0.80
Control	20	11	0.55
IGF-1	20	14	0.70
BIO	20	17	0.85
BIO and IGF-1	20	16	0.80

Histology and immunohistochemistry

At 6 weeks post-implantation, the rats were anesthetized by 3% pentobarbital sodium (30 mg kg^{-1}) and then arrested with 10% KCl, rapidly excised, and frozen in tissue medium, which was followed by sectioning each heart into three or four transverse slices parallel to the atrioventricular ring. Samples were then fixed with 10% buffered formalin, embedded in paraffin, sectioned into 5 μm slices, and stained with hematoxylin and eosin (H&E). Scar thickness was measured under a microscope, and on this basis, the relative scar thickness (defined by the average scar thickness divided by the average wall thickness) and the heart expansion index (defined by the LV cavity area/whole LV area/relative scar thickness) were evaluated, as in our previous study [104]. The detection of various tissue antigens was conducted using the primary antibody, CD31, for endothelial cells (Abcam plc). Double staining with anti-cardiac troponin-T (green, cardiomyocyte marker; Abcam plc) and anti-PCNA (red, proliferation marker; Abcam plc) was performed. The doubled-stained cells were evaluated by counting the average number of positively stained cells within six areas of $0.1\,\mathrm{mm}^2$. The CD31-positive capillary densities at the infarcted areas were analyzed by employing the same method.

Statistical analysis

All of the results were reported as the mean $\pm$ standard deviation. Two-way analysis of variance (ANOVA) was used in the analysis of heart function between groups. One factor is the revascularization and the other is cardiomyocytes regeneration. Post hoc Student's t-test was used for the remainder of the analysis to identify which particular differences between pairs of means are significant. A P-value <0.05 (*) was considered significant; a P-value <0.01 (**) was very significant.

2.4 Results

2.4.1 Preparation and Characterization of Hydrogels and the Drug-release Profile *in vitro* and *in vivo*
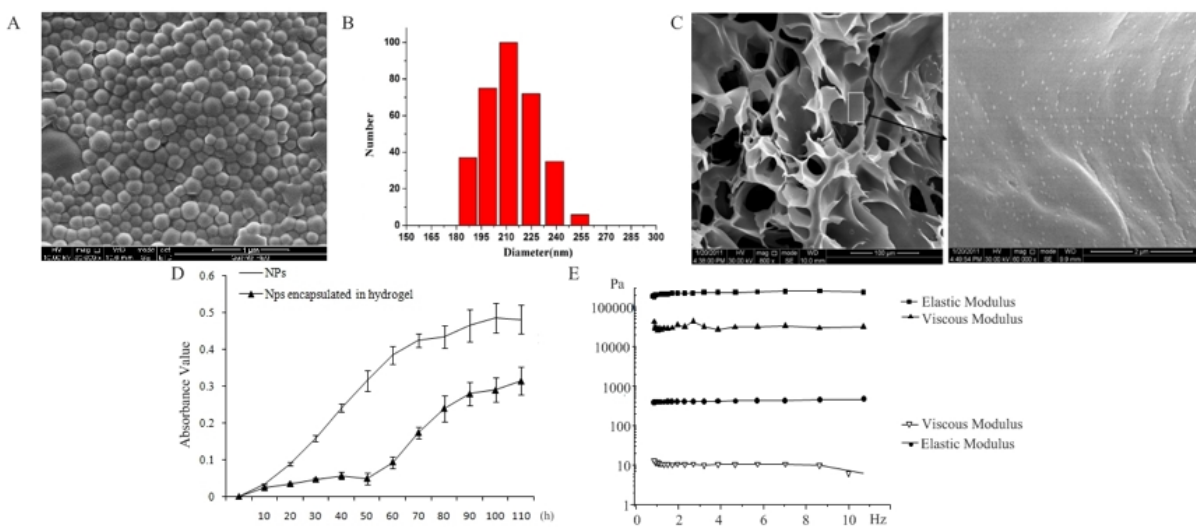
Figure 2.1: Release assay profile in vitro. Notes: (A) SEM morphology of the prepared gelatin NPs. (B) Dynamic light scattering (DLS) analysis of the gelatin NPs. (C) SEM image and a zoomed image showing the gelatin NPs covalently conjugated to the hydrogel. (D) The UV results of BIO absorption showing the release profiles in the free NP group and in the gelatin NP encapsulated in hydrogel group. (E) Mechanical properties of the hydrogel before and after release. Abbreviations: SEM, scanning electron microscope; DLS, dynamic light scattering; BIO, 6-bromoindirubin-3-oxime; NP, nanoparticle; UV, ultraviolet.

The morphology of BgNPs was observed using a scanning electron microscope (SEM) (Figure 2.1 A); the results of dynamic light scattering showed that the NPs formed a cluster featur-

ing different diameters ranging from 180 nm to 255 nm with a polydispersity index (PDI) of 0.158 ± 0.029 (Figure 2.1B). Then, the NPs were covalently attached to the hydrogel, as we had previously reported [102]. The hydrogel formed with the porous network structure (Figure 2.1C). The loading efficiency of OgNPs and FgNPs, which contained BIO and IGF-1, respectively, were about 55% or 60%, which is similar to the findings of a previous study [107]. To detect the release profile of the NPs and the hydrogel composite system, we designed a method to examine the BIO release profile in the NPs conjugated to the hydrogel and in the free NPs, as described in the encapsulation efficiencies of NPs section. We compared the release behaviour of BIO in NPs and in NPs covalently conjugated to the hydrogel. The release profile assay lasted for 110 hours for both groups. The release rate of BIO in NPs was much higher than that the release rate of BIO in NPs encapsulated in hydrogel (Figure 2.1D). From 0 to 60 hours, a rapid increase in the drug release was observed in the NPs; however, a similar release peak was detected from 60–90 hours in the NPs conjugated to the hydrogel (Figure 2.1D). For NPs, the BIO release mechanisms included the biodegradation of NPs; in addition, BIO diffuses to the solution outside the semi-permeable membrane.

In contrast with NPs, two steps were involved in the BIO's release profile in NPs covalently conjugated to the hydrogel. The first step involves the breakage of amines found between the NPs and the hydrogel, and the second step includes BIO's release from the NPs. As a result, the peak release was postponed 60 hours in the in vitro delivery system. To further confirm the release mechanism of NPs, the mechanical properties of the hydrogel were measured and compared to its original properties prior to being released. The results showed that the elastic and viscous moduli of the hydrogel were around 10^5 and 10^4 Pa prior to release, respectively, and they had decreased to 10^3 and 10 Pa, respectively, after release (Figure 2.1E).

However, there was a tendency where the elastic and viscous moduli to stay the same before and after the release, which indicated that the hydrogel still maintained a gel network structure. Therefore, we postulated that the main releasing mechanism occurs through the breakage of chemical bonds between gelatin NPs and oxide alginate–gelatin hydrogel. The release profile was also controlled by the cross-linking density and degradability of the gel, modulated by the initial gelation condition. The *in vivo* release profile was evaluated using the *in vivo* imaging method, as shown in Figure 2.2 A. The results illustrated that the fluorescence of the scaffold encapsulating Cy7-labeled

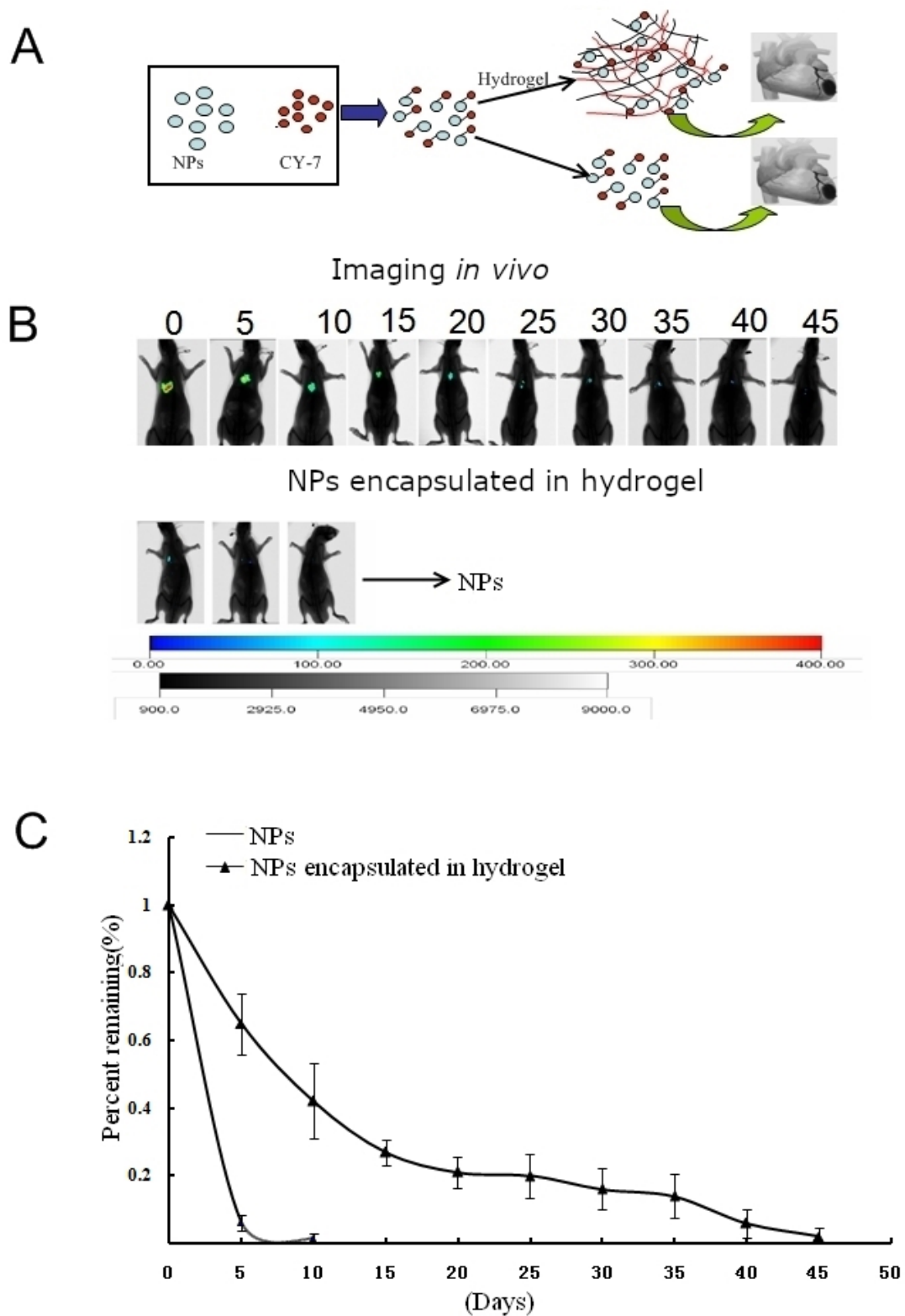**Figure 2.2:** *In vivo* release profile assay. Notes: (A) The scheme showing the strategy used to monitor the release profile using an *in vivo* imaging method. (B) Typical images of rats implanted with free Cy7-labeled NPs and Cy7-labeled gelatin NPs encapsulated in hydrogel. (C) The statistical analysis of immunofluorescence intensity remained constant over time (n6). Abbreviation: NP, nanoparticle.

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NPs could last for 45 days, while the fluorescence of the control group (free Cy7-labeled NPs, which were not conjugated to the hydrogel) lasted for less than 10 days (Figures 2.2 B and 2.2 C). Moreover, the intensities of the fluorescence were much higher than those in the control group (Figure 2.2 C).

2.4.2 Cardiomyocyte Proliferation Detected in Hydrogels

To mimic the *in vivo* effect of the drug delivery system among cardiac cells, a three-dimensional (3D) system was used to encapsulate the cardiomyocytes in hydrogel in the present study. The results showed that the percentage of proliferated cardiomyocytes in the BIO release system ($15\% \pm 4\%$) was significantly higher than that in the control groups ($7\% \pm 3\%$; $P < 0.05$); however, no significant difference was observed in the IGF-1 release group ($11\% \pm 2\%$), as shown by the laser confocal images (Figures 2.3 A, 2.3 B, and 2.3 C) and the statistical analyses (Figure 2.3 D). Therefore, IGF-1 could not induce the proliferation of cardiomyocytes.

2.4.3 BIO/IGF-1 Administration Improves Cardiac Function after MI and Leads to the Formation of New Cardiomyocytes

MI animal models were prepared according to the method described above, and different hydrogels that covalently conjugated with various NPs (blank gelatin NPs, OgNPs, FgNPs, and CgNPs) were implanted to detect their effects on cardiac function repair. Even though IGF-1 by itself was ineffective in promoting cardiomyocyte proliferation in a culture system, there might be integrated impact between BIO and IGF-1 for MI repair.

In order to study the mechanism underlying the advancement of cardiac function for the administration of BIO + IGF-1 in the MI rats, H&E staining was used perform a histology evaluation following injection with our hydrogel system. Cardiac remodeling was found in our experimental groups; however, through visual inspection, when compared to the control group, more cardiomyocytes can be found visually in the BIO + IGF group as shown in Figure 2.4. Note that even though fibrosis can be observed in the slides, it is commonly not used as an evaluation factor in this type of research. The results were further confirmed by double staining with the anti-cardiac troponin-T (green; cardiomyocyte marker) and anti-PCNA (red; proliferation marker) assay. The number of
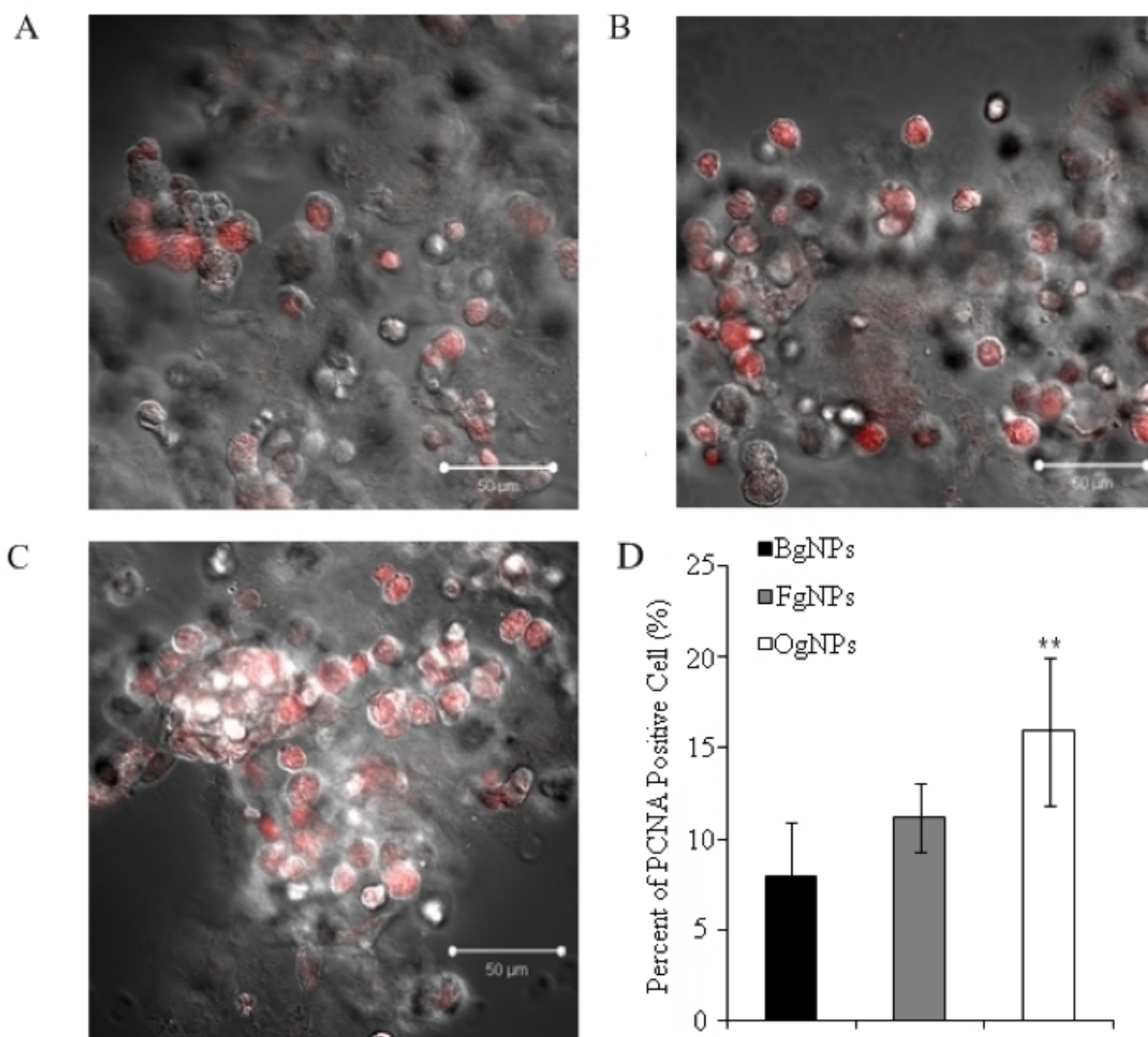
Figure 2.3: Effect of the hydrogel delivery system on the proliferation of 3D culture cardiomyocytes. Notes: Scheme showing the strategy used to detect the enhanced proliferation of 3D-cultured cardiomyocytes. Laser confocal images showing the PCNA-positive stained cells in the (A) BgNP group, (B) FgNP group, and (C) OgNP group. (D) Statistical analysis (t-test) of the number of PCNA-positive cells (n=6). $**P = 0.01$ OgNPs versus the BgNP group. Abbreviations: BIO, 6-bromoindirubin-3-oxime; 3D, three dimensional; PCNA, proliferating cell nuclear antigen; NP, nanoparticle; BgNP, blank gelatin nanoparticle; OgNP, BIO-loaded gelatin nanoparticle; IGF-1, insulin-like growth factor 1; FgNP, IGF-1-loaded gelatin nanoparticle.
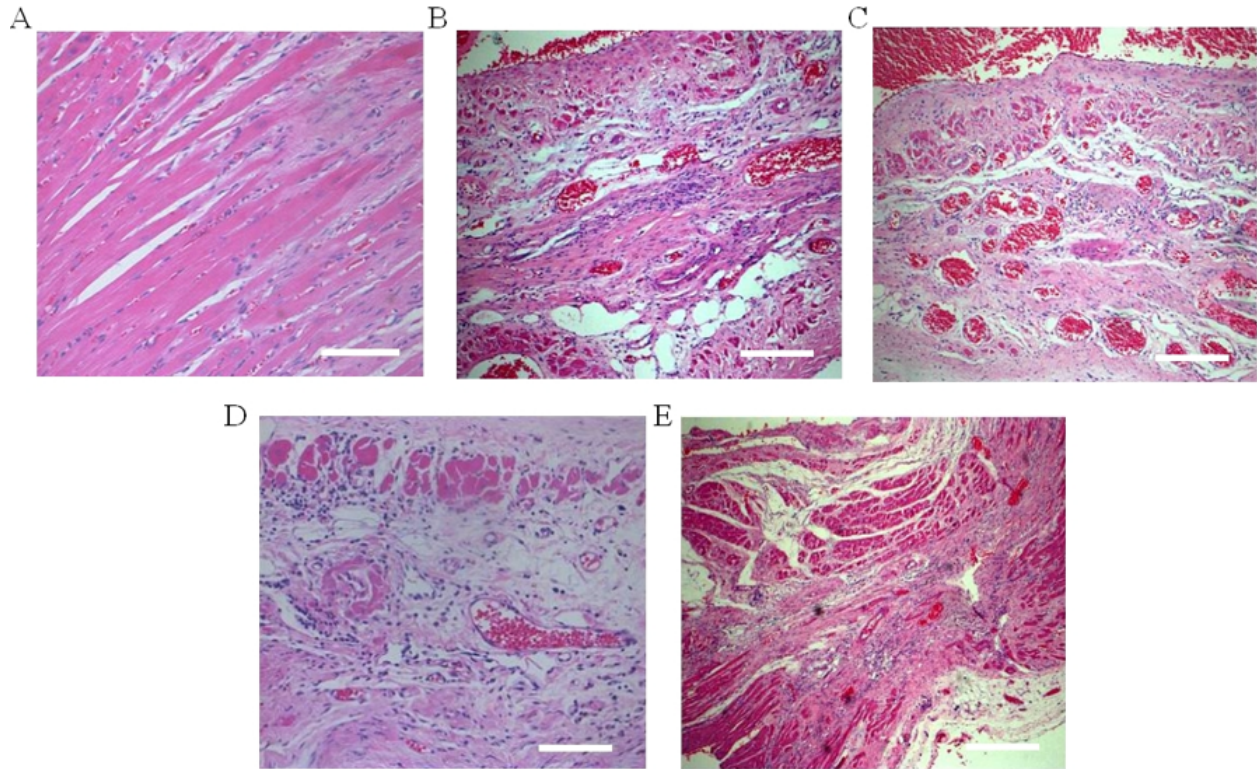
Figure 2.4: Histology evaluation of an ischemic heart. More cardiomyocytes can be found visually in (E) as compared to the others. Notes: (A–E) H&E staining of the tissue sections obtained from the MI region among the (A) sham group, (B) control group, (C) IGF-1 release group, (D) BIO release group, and (E) BIO and IGF-1 co-delivery group. Scale bar, 100um. Abbreviations: BIO, 6-bromoindirubin-3-oxime; IGF-1, insulin-like growth factor 1; H&E, hematoxylin and eosin; MI, myocardial infarction.

proliferating cardiomyocytes in the BIO + IGF-1 group (35 ± 5) was the most significant among all groups, and the number of proliferating cardiomyocytes in the BIO group (27 ± 5) was also significantly higher than that of both the sham (5 ± 2) and the control groups (15 ± 5) as shown in Figure 2.5. In the BIO delivery group, the enhanced proliferation of cardiomyocytes was observed. In the co-delivery of BIO + IGF-1 group, both enhanced proliferation of the cardiomyocytes and function recovery were seen. Moreover, angiogenesis plays a crucial role in tissue repair and heart function after MI; therefore, the CD31 antibody-positive angiogenesis assay was detected among the different groups as shown in Figure 2.6. In the BIO+ IGF -1 group, the most capillaries ($70 \pm 8/$ mm^2) was observed. In addition, significant increase of blood vessels ($60 \pm 7/mm^2$) in the IGF-1 group was also detected. However, no significant increase in blood vessel density was observed in the BIO group ($43 \pm 8/mm^2$), which suggested that BIO could not promote angiogenesis. Enhanced

angiogenesis was accompanied by functional recovery in the BIO and IGF-1 group; this indicates that angiogenesis may be an important factor in the recovery of function.
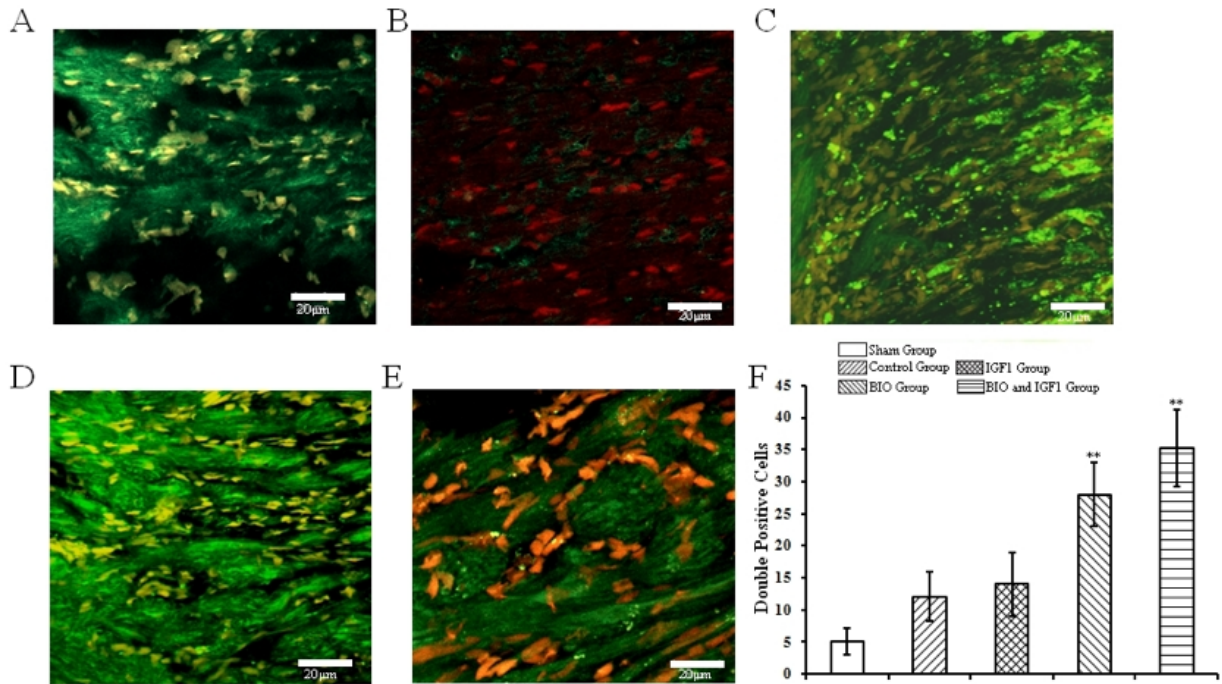
**Figure 2.5:** Double staining with anticardiac troponin-T (green; cardiomyocyte marker) and anti-PCNA (red; proliferation marker) shows the enhanced proliferation of resident cardiomyocytes in an MI model (P< 0.01; n=6). Notes: (A) Sham group, (B) control group, (C) IGF-1 release group, (D) BIO release group, and (E) BIO and IGF-1 co-delivery group. (F) Statistical analysis of the number of double-stained cells. **$P < 0.01$ versus the control group. The arrows show the cardiomyocytes which are double staining. Abbreviations: BIO, 6-bromoindirubin-3-oxime; IGF-1, insulin-like growth factor 1; MI, myocardial infarction; PCNA, proliferating cell nuclear antigen.

2.5   Discussion

In the present study, we investigated the functional and histological/cellular effects of the intramyocardial administration of BIO and IGF-1 in MI rats. We showed that the improved sustained co-delivery of BIO and IGF-1 by a newly developed hybrid hydrogel system holds potential as a novel treatment for MI. Six weeks after delivery, BIO and IGF-1 treatment led to the proliferation of resident cardiac cells and promoted revascularization. Importantly, the repair and regeneration of the damaged myocardial tissues was associated with significant improvements in cardiac function
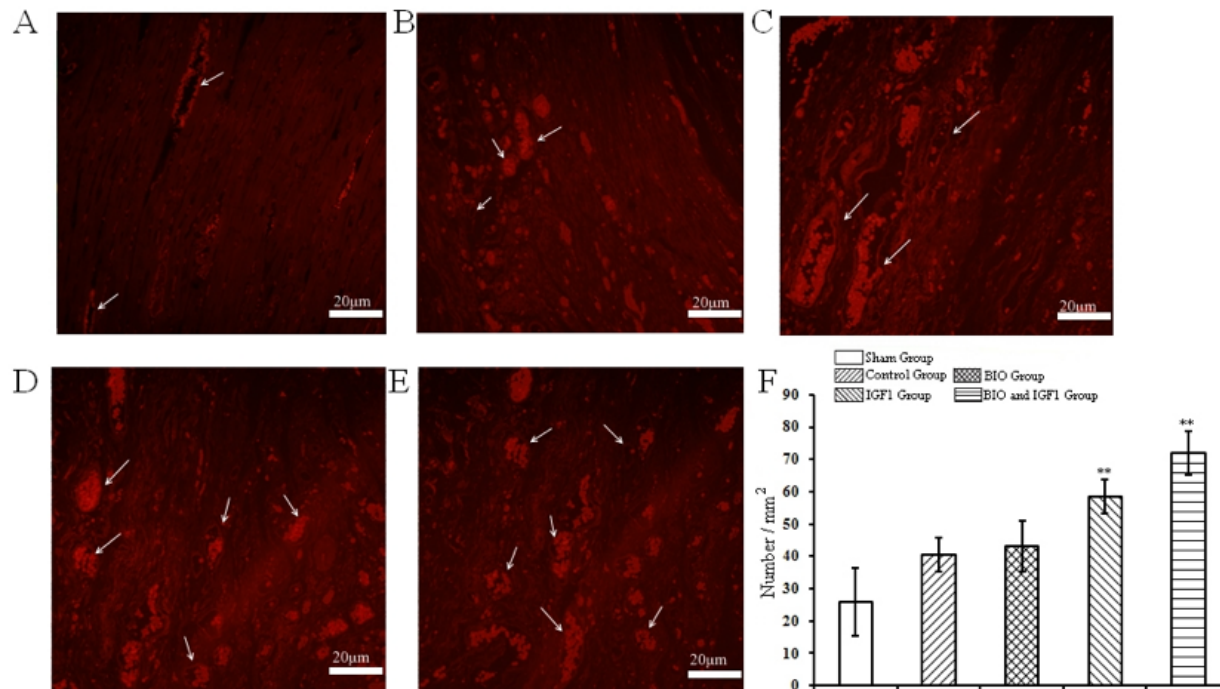
Figure 2.6: Immunostaining for the evaluation of angiogenesis. Notes: (A–D) CD31 immunostaining of the tissue sections from the MI among the (A) sham group, (B) control group, (C) BIO release group, (D) IGF-1 release group, and (E) BIO and IGF-1 co-delivery group. (F) Statistical analysis of the CD31-positive blood vessels ($n = 6$). $*P < 0.05$ versus the control group; $**P < 0.01$ versus the control group. Abbreviations: BIO, 6-bromoindirubin-3-oxime; IGF-1, insulin-like growth factor 1; MI, myocardial infarction.

as shown in Figure 2.7.

2.5.1 Development of a New Hybrid Hydrogel System

Recently, studies have reported on the ways in which cardiac diseases can be treated through the sustained delivery of IGF-1 by means of hydrogels [108, 109]. Though it makes a difference in the treatment of MI, IGF-1 also causes such issues as toxicity due to their high doses and the short release profile. Gelatin NPs, due to their good biocompatibility and biodegradability, have been widely utilized in drug delivery applications [110]. However; the relatively fast degradation of gelatin limits its applications to long-term protein delivery. Our previous study showed that the covalent cross-linking of gelatin with alginate forms a hydrogel with both enhanced mechanical properties and bioactive motifs for cell attachment, biodegradability, and functional recovery [104].
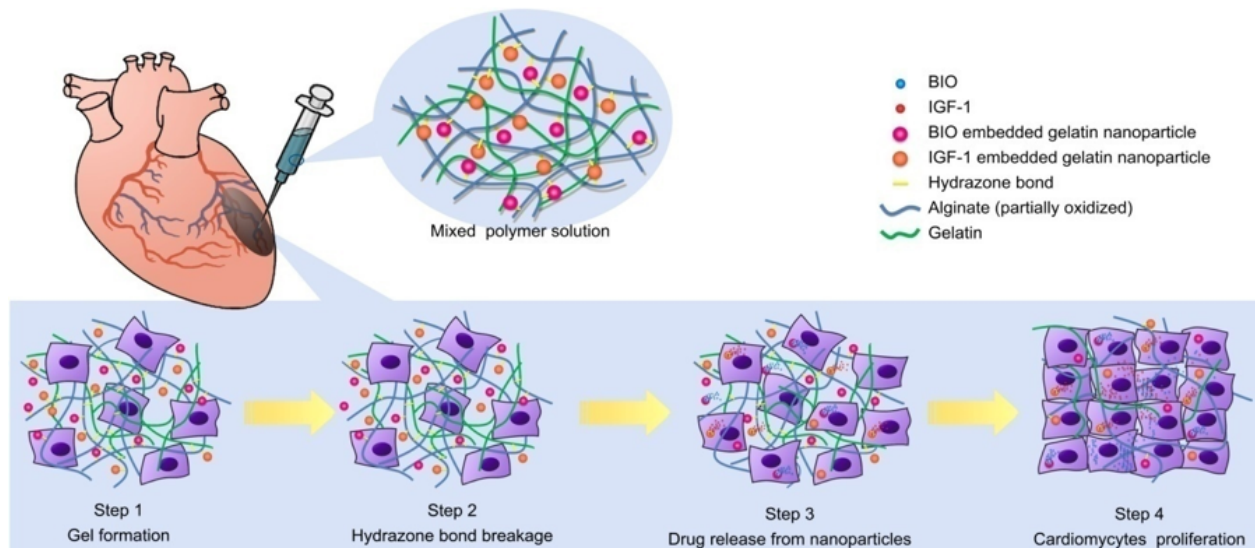
Figure 2.7: Scheme showing the mechanism of BIO release within the hydrogel after injection into the MI area. Abbreviations: BIO, 6-bromoindirubin-3-oxime; IGF-1, insulin-like growth factor 1; MI, myocardial infarction.

In the current study, we developed a novel hybrid hydrogel system with chemically encapsulated gelatin NPs, which are pH sensitive, for the sustained co-delivery of BIO + IGF-1 in MI treatment. The oxidation of sodium alginate produced aldehyde groups, which can form unstable amines found within the free amino group in the gelatin NPs; thus, they covalently linked the NPs to the alginate polymer chain. An acidic, low pH environment was generated with the occurrence of an MI [83]. The amines that were found, which form between alginate and the gelatin NPs, are most active in acidic environments. Following MI, the bond broke down quickly and the BIO and IGF-1 were released to the damaged area of the heart, ultimately promoting the heart's overall function.

In summary, in our study, we developed a novel, pH-sensitive, hybrid hydrogel system of chemically encapsulated gelatin NPs that can more efficiently sustain the co-delivery of BIO and IGF-1 to the injured area of the heart. Further, we also illustrated that the system could be more advantageous when delivering BIO and IGF-1 in the treatment of MI.

2.5.2 Sustained Release of BIO or IGF-1 using Hybrid Hydrogel Systems

Drug release profiles were detected both in vitro and *in vivo*. It is generally recognized that 2D cell cultures are not adequate representatives of the cellular environment found in organisms; rather, 3D cell culture models have the potential to improve the physiological relevance of cell-based

assays [111]. In our previous study, we constructed an engineered heart tissue model using cardiomyocytes [104]. In order to mimic the *in vivo* effect of a drug delivery system when promoting the proliferation of cardiac cells, a 3D system to encapsulate the cardiomyocytes in the hydrogels was used in the present study. The drug-release rate was detected *in vivo* by an imaging system that has been widely used in cancer studies. More recently, a study showed that it can also be used to evaluate biomaterial biodegradation *in vivo* [112]. Cy7 was conjugated to the NPs, and the rapidly decreasing fluorescence intensities in the NP group indicated that without the support of the substrate, the NPs diffused quickly following injection into the infarcted area. Also, it was found that the fluorescence intensities decreased quickly at first, and then they slowly continued to decrease with the treatment of MI via the injection of hydrogels; this result may be due to the fact that our system is pH sensitive. Our results on the elastic and viscous moduli of the hydrogel illustrate that there is not much difference prior to and after release, which indicates that the hydrogel still maintains a gel network structure. As such, the controlled release profile was mainly caused by the breakdown of the chemical bond between NPs and hydrogel, rather than by the drug itself.

2.5.3 Heart Regeneration with Our Novel Hybrid Hydrogel System of Chemically Encapsulated Gelatin NPs

With the development of intramyocardial biomaterial, more and more biomaterials are being used for injection therapy [108]. To date, it has already been demonstrated that many small molecules contribute to heart regeneration; however, the mechanisms by which these benefits are obtained remain unclear [113]. Here, we show that following BIO administration, the number of resident cardiac cells in an MI model increased. Our findings further indicate that the function of BIO can promote dedifferentiation and proliferation in terminally differentiated cardiomyocytes *in vivo*. The BIO could reprogram the cardiomyocytes with changed morphology evidenced through rearrangement of the cytoskeleton [93], which can be clearly observed in the anticardiac troponin-T staining in Figure 2.5. In a recent study by Kohler, it was demonstrated that low doses of BIO also have the ability to induce partial dedifferentiation among endothelial cells by regulating the canonical Wnt pathway [114]. A previous study has shown that there is a dual role for BIO, which involves the maintenance of stem cell properties and the induction of proliferation in differentiated cardiomy-

ocytes; this indicates that these functions may share common molecular pathways that control the canonical Wnt pathway [93, 115]. Secondly, our present findings document that IGF-1 can induce angiogenesis following MI. Consistent with our study, a previous study showed that the local delivery of the IGF-1 gene by recombinant adeno-associated virus in the setting of acute MI resulted in sustained IGF-1 expression, increased angiogenesis, and improved cardiac function [116]. Another study also showed that IGF-1 enhanced wound healing and induced angiogenesis via a vascular endothelial growth factor (VEGF)-independent pathway [117]. A previous study showed that immobilizing IGF-1 with hydrogel could serve as a controlled drug-release platform, and that it could also enhance cardiac progenitor cell survival and differentiation. In a study on chronically infarcted pig hearts, IGF-1 and hepatocyte growth factor therapy also resulted in improved cardiac function when using hydrogels [109]. In our current research, we developed a novel hybrid hydrogel system with chemically encapsulated gelatin NPs for the sustained co-delivery of BIO and IGF-1 in MI treatment. To our knowledge, this is the first report that remedies MI via the co-delivery BIO and IGF-1, which have been identified as potential candidates to guide postnatal stem progenitor cells toward a cardiomyogenic fate. In the present study, we demonstrated that the co-delivery of BIO and IGF-1 can promote resident cardiomyocyte proliferation and revascularization. Therefore, both BIO and IGF-1 can be involved potentially in contributing to the heart's functional recovery. As such, the entire study protocol employed in this present work is clinically feasible and may be performed in a conventional catheterization laboratory.

2.5.4 Limitations

The concentrations of BIO and IGF-1 that were used in this work were adopted and used as per previous studies [93, 100]. In the present study, the co-delivery of these two agents promoted the function and regeneration of the heart following MI. However, it is not clear if the optimal concentration of the co-delivery of BIO and IGF-1 can play an even more significant role in improving heart function after MI, which would be interesting to pursue in the future. In addition, although experimental work on the *in vivo* drug release of NPs encapsulated in hydrogel showed a 45-day release for Cy7, we can not detect the release efficiency of BIO and IGF-1 directly. It would be interesting to observe the delivery of these drugs over a longer period of time. This line of inquiry also suggests that more non-invasive methods should be developed for drug tracing in the future.

In section 2.4.3, the weight of the heart relative to body weight at the time of euthanasia was not measured and this should be incorporated in the future experiments.

2.6   Conclusions

A novel hydrogel delivery system was developed to deliver drugs specifically to the site of an MI to selectively enhance the proliferation of resident cardiac cells, resulting in enhanced heart function recovery. Our results showed that NPs covalently entrapped in hydrogel hold great promise for treating MIs and other related diseases given their ability to deliver a wide range of therapeutics.

3 Synthesis of Injectable Alginate Hydrogels with Muscle-Derived Stem Cells for Potential Myocardial Infarction Repair

This chapter has been published as "Fang, Rui, Weiming Tian, and Xiongbiao Chen. "Synthesis of injectable alginate hydrogels with muscle-derived stem cells for potential myocardial infarction repair." Applied Sciences 7.3 (2017): 252." According to the Copyright Agreement, "the authors retain the right to include the journal article, in full or in part, in a thesis or dissertation."

3.1   Abstract

Myocardial infarction (MI), caused by the occlusion of the left ventricular coronary artery, leads to the loss of cardiomyocytes and, potentially, heart failure. Cardiomyocytes in adult mammals proliferate at an extremely low rate and thus, a major challenge in MI treatment is supplementing exogenous cells and keeping them viable in MI areas. To address this challenge, injecting hydrogels encapsulating cells into MI areas, to compensate for the loss of cardiomyocytes, shows promise. This study synthesized two types of alginate hydrogels, based on self-crosslinking (SCL) and calcium ion crosslinking (Ca^{2+}) in varying formulations. The hydrogels encapsulated living muscle-derived stem cells (MDSCs) and their performance was evaluated in terms of optimizing cell viability during the injection process, as well as the live/dead cell rate after long-term cultivation. The morphology of the hydrogel-encapsulated cells was characterized by scanning electronic microscopy (SEM) and live/dead cells were examined using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide staining) assay. The mechanical properties of the hydrogels were also determined via a rheometer, to identify their influence on cell viability during the injection process and with respect to long-term cultivation. The SCL hydrogel with a 0.8% alginate and 20%

gelatin formulation resulted in the highest cell viability during the injection process, and the Ca^{2+} hydrogel composed of 1.1% alginate and 20% gelatin maintained the highest cell survival rate after two months in culture.

3.2   Introduction

Myocardial infarction (MI), commonly known as a heart attack, is caused by an interruption of the blood supply to a part of the heart with cardiovascular disorder [118]. If left untreated, the ischemia or resulting restriction in the blood supply may cause damage to the myocardium or cardiomyocyte apoptosis, thus leading to heart failure. According to the latest statistics from the American Heart Association [119], MI and other cardiovascular diseases are the leading causes of death worldwide. The heart is primarily composed of the myocardium and coronary arteries. Supplied with oxygen- and nutrient-rich blood through the coronary arteries, the myocardium continuously contracts and relaxes, to pump blood throughout the body. The myocardium has an extremely limited regeneration potential [120, 121], and so, after MI, the ischemic myocardium ceases to function properly, due to the loss or death of cardiomyocytes, i.e., the cells of which it is comprised. Cardiomyoplasty is a therapy used in MI repair, in which cardiomyoblasts or other types of stem cells are directly injected into the injured heart, serving as renewable cellular sources which aid in the repair of the damaged myocardium [122, 123]. To compensate for damaged or dead cardiomyocytes in the MI area, the transplantation of various types of stem cells or exogenous cardiomyocytes has been studied. Among them, cardiomyocytes [124, 125], embryonic stem cells [126, 127], and bone marrow-derived mesenchymal stem cells [128] have been investigated, but the ideal cell type for MI repair is far from certain [129]. Muscle-derived stem cells (MDSCs) are a promising type of cell for MI repair because they have the ability to trans-differentiate into cardiomyocytes when co-cultured with cardiomyocytes [130], resist oxidative stress-induced apoptosis [131] , reduce scar formation [131, 132], and improve cardiac function [133, 134] after transplantation. Although cardiomyoplasty is promising for MI repair, low cell viability after transplantation, and then gradually decreasing cell survival after long-term cultivation, are issues that remain to be addressed. Encapsulating the cells to be transplanted into MI hearts in synthesized hydrogels may be an effective way to improve the cell survival rate. Scaffolds or engineered constructs are important for tissue

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engineering [135], especially those with bioactive growth factors as a delivery system, which are of great importance for cardiovascular tissue engineering [136]. The co-release of growth factors of VEGF (vascular endothelial growth factor), bFGF (basic fibroblast growth factor), and PDGF (platelet derived growth factor) from engineered constructs for cardiac repair after MI is promising [137], but many conditions, such as the release rate, need to be regulated.

Hydrogels are cross-linked networks of polymers, such as fibrin and alginate, with a high water content between polymer chains [138, 139]. When used for cell encapsulation, hydrogels provide a hydrated tissue-like environment for cell attachment and protection, thereby improving cell viability. Hydrogels can provide biochemical cues and structural support, along with the possible recruitment of endogenous stem cells [140]. Cellular growth and functions are critically affected by both the cell compatibility of materials [141], and the pore sizes in the hydrogel structure [142], which lead to varying cell viabilities. Combining injectable microspheres with a hydrogel is an alternative approach [143]; however, research into cell therapies integrating microspheres and hydrogels has been limited [144]. Among various injectable hydrogels, alginate cross-linked in the presence of calcium ions has been drawing considerable attention for use in MI repair and can lead to enhanced cell viability and proliferation, as well as an improved contractile function [145, 146]. Alginate, a biodegradable and biocompatible polysaccharide commonly found in algae, was employed in this study, due to its successful use as a hydrogel for tissue engineering [147, 148]. However, one inherent disadvantage of alginate is the lack of cell-surface receptors for cell attachment, resulting from the negatively charged polymer. Considering this, we also used gelatin to encourage cell adhesion, as it is the main component of the extracellular matrix (ECM) and can promote cell attachment and migration [149, 150]. This research mainly focuses on optimizing (i) cell viability during the injection process and (ii) the live/dead cell ratio after a period of cultivation by optimizing the composition of alginate hydrogels encapsulating living MDSCs for potential MI repair.

3.3 Materials and Methods

The preparation of materials for the experiments presented in this paper was carried out according to protocols approved by the University of Saskatchewan Ethics and Integrity in Animal Research

Committee (Saskatoon, Canada) and the Harbin Institute of Technology's Animal Care (Harbin, China) and Use Committee.

3.3.1 Materials

Sprague Dawley (SD) rats (m=400g, Laboratory Animal Center, Harbin Medical University, China), Muscle derived stem cells (MDSCs, Bio-X center, Harbin Institute of Technology), Dulbecco's Modified Eagle Medium (Boster, Wuhan, China), 10% horse serum (Boster, Wuhan, China), sodium alginate (low viscosity, Sigma, St. Louis, USA), semipermeable membrane (40 KDa, Sigma, St. Louis, USA), MTT cell proliferation kit I (Sigma, St. Louis, USA).

3.3.2 Methods

Gelatin Extraction from Rat Tails

The gelatin used as a component of both hydrogels was obtained from the tails of Sprague Dawley (SD) rats, following the same procedure as was presented in our previous work [151]. Briefly, three male SD rats (400 g) were anaesthetized and collagen from their tails was extracted stick-by-stick, via forceps. The collagen was dissolved in 0.5% glacial acetic acid (50 mL) at $4\,^\circ$C for $72\,$h. Then, the mixture (solution) of collagen and glacial acetic acid was sterilized by membrane filtration (0.28 um) and finally freeze-dried to form a powder. The concentration of gelatin in this study was determined by measuring the mass/volume ratio of the mass of collagen that dissolved completely in the glacial acetic acid, to the volume of the solution. The gelatin powder was dissolved in sterile phosphate buffer solution (PBS) to a concentration of 40%, for synthesizing the hydrogels in this study.

Cell Cultivation

Using the same technique employed for 3D cell cultivation as in our previous work [146], MDSCs were cultured in the two types of hydrogels described in sections 2.3 and 2.4. Briefly, MDSCs were mixed evenly with alginate and then cross-linked with gelatin to form hydrogels. After the crosslinking process, the cells were encapsulated in the hydrogels and then were cultured in Dulbecco's Modified Eagle Medium (DMEM, 10% horse serum added), which was refreshed daily.

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SCL Hydrogel Synthesis

SCL hydrogels were synthesized with partially oxidized sodium alginate cross-linking with gelatin, following our previously published procedures [152]. Four hydrogel formulations, namely SCL 0.8%, SCL 1.1%, SCL 1.5%, and SCL 2%, based on their respective sodium alginate concentrations, comprised four experimental groups. For each experimental group, sodium alginate (low viscosity, Sigma, St. Louis, USA) was oxidized by sodium periodate at a concentration ratio of 2:1 for 8 h at 4 $\circ C$ in the dark. The oxidation degree was measured via the mass of the remaining sodium peroxide. Briefly the oxidization process was described as following: Sodium chloride (0.5 g) and 100% ethanol (10 mL) were added to the oxidized alginate solutions, until sediments formed. The sediments were then dissolved in deionized water (10 mL) and dialyzed for 72 h with a semipermeable membrane (40 KDa, Sigma) at 4$\circ C$; thereafter, they were freeze dried into powder and dissolved in sterile PBS to a concentration of 60%, and were then mixed with MDSCs to a density of 0.5 x 10^5/mL. Sodium alginate solutions containing MDSCs were mixed with gelatin at a 1:1 volume ratio and then printed through a 0.26 mm needle into hydrogels (beads). These hydrogel-encapsulated cells were cultured in DMEM (refreshed daily) in a cell culture incubator for two months.

Ca^{2+} Hydrogel Synthesis

Ca^{2+} hydrogels were synthesized via the physical crosslinking between calcium ions and alginate, that is based on the attraction between positive and negative charges. Four hydrogel formulations, namely Ca^{2+} 0.8%, Ca^{2+} 1.1%, Ca^{2+} 1.5%, and Ca^{2+} 2%, based on their respective concentrations of alginate, were prepared and represented four experimental groups. For each experimental group, MDSCs (0.5 x 10^5/mL) were mixed with sodium alginate solution and then with gelatin, at a 1:1 volume ratio. The mixture was dispensed through a 0.26 mm needle into 100 mM calcium chloride solution (1% tween-20 added), in a tissue culture dish. Hydrogels (beads) encapsulating cells were formed at the moment the mixture entered the calcium chloride solution. The calcium chloride was then removed and the hydrogels were quickly rinsed with PBS. Cells were then cultured in the same environment as outlined in section 2.3, for two months.

SEM Examination of Hydrogel Morphology

The structure of the hydrogel-encapsulated MDSCs for each experimental group was examined via scanning electron microscopy (SEM, Quanta 200, FEI, Hillsboro, USA). First, the hydrogels were fixed in a solution containing 2% glutaraldehyde and 3% formaldehyde in cacodylate buffer (0.1 M cacodylate, 0.09 M sucrose, 0.01 M $MgCl_2$, and 0.01 M $MgCl_2$, pH 6.9), for 1 h. They were then dehydrated with a graded series of acetone (10%, 30%, 50%, 70%, 90%, and 100%) on ice, with each lasting 15 min, followed by critical point drying with liquid CO_2. Last, the hydrogels were each cut into two hemispheres and attached to a conductive adhesive, then sputter coated with a gold film to a thickness of 10 nm for observation via SEM (Quanta 200, FEI, Hillsboro, USA).

Mechanical Strength Measurement

The mechanical strength of both types of hydrogels was analyzed via rheometry, conducted at a sweeping frequency ranging from 0.1 to 10 Hz. Hydrogels of a larger size were synthesized with the same technique described in sections 2.3 and 2.4, via a pipette (1000 uL) in a six-well culture dish. The hydrogels can form a lamella that fills up one whole well of the dish. Lamellas formed from Kingdom), with a CP40 cone.

Hydrogel Degradation

Hydrogels were synthesized with an original weight of 0.8 g (SCL hydrogels) and 0.5 g (Ca^{2+} hydrogels) in each group (n = 6), and were then exposed to 0.5% collagenase type II (5 mL) at body temperature, for 48 h. The hydrogels were then freeze-dried into powder for the weight measurement. This process was repeated until all of the hydrogels had degraded (i.e., disappeared completely).

MTT Assay for Cell Proliferation

The MDSCs encapsulated in all groups of both types of hydrogels were released by dissolution in 3% sodium citrate, after 2 h, 24 h, and two months of cell cultivation. Then, the survival rate of these cells was measured by a MTT assay with the cell proliferation kit I (Sigma), immediately after the cells were released from the hydrogels. Briefly, cells from each group were coated with

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide staining) for 4 h in 96-well culture dish plates, until sediments formed; the control group was DMEM coated with MTT. The three same wells for used for each group and each test was repeated six times. Then, with MTT dissolvent was kept for another 4 h in the cell culture incubator. The optical density (OD) of the resulting solution was determining using an ultraviolet spectrophotometer.

Fluorescent staining for Live/Dead Cells

MDSCs were released from the hydrogels of each experimental group via the method described in section 2.8, after 2 h, 24 h, and two months of cultivation. They were then stained with calcein (green) for 20 min and PI (red) for 5 min, to label live and dead cells, respectively. MDSCs were also stained with DAPI, to mark the cell nuclei. The fluorescent staining in each layer of the hydrogels was examined using confocal microscopy (FV3000, Olympus, Tokyo, Japan).

3.4   Results and Discussion

3.4.1   Morphology

The morphology via SEM is shown in Figure 3.1 , illustrating the texture and inner structure of the SCL and Ca^{2+} hydrogels of all groups. Specifically, it shows the morphology of hydrogels without cells: 1A for the SCL hydrogel and 1a for the Ca^{2+} hydrogel; and the morphology of hydrogels with cells: 1B, 1C, 1D, and 1E SCL hydrogels, and 1b, 1c, 1d, and 1e for Ca^{2+} hydrogels, where the alginate concentrations are 0.8%, 1.1%, 1.5%, and 2%, respectively. The SEM images show no major differences in the inner structure of both SCL hydrogels and Ca^{2+} hydrogels, but reveal minor differences between the two types of hydrogels in terms of pore size and degree of compactness. Specifically, when studying the software image J, for the SCL and Ca^{2+} hydrogels, the average areas of pore size were found to be 124 μm^2 and 7.1 μm^2, respectively, which indicates that the pore size of the SCL hydrogels is significantly larger than that of the Ca^{2+} hydrogels; and the average number of pores in each type of hydrogel per 125 μm^2 area was six and 89, respectively, indicating the large number of pores in Ca^{2+} hydrogels. When considering the pore density, calculated as the overall area of pores (pore size multiplied by pores amount per 125 μm^2) of each type of hydrogel, it was

found to be slightly higher for the SCL hydrogels (744 µm^2) than for the Ca^{2+} hydrogels (632 µm^2). The overall structure of the SCL hydrogels is a connective network, which contains fewer pores, but of a larger size, than the Ca^{2+} hydrogels; while the Ca^{2+} hydrogels reveal a more compact network structure.

With respect to the ability of the two types of hydrogels to entrap cells, the SEM images show that the Ca^{2+} hydrogels, with their larger number of pores, appear better able to encapsulate cells than the SCL hydrogels. Indeed, it was noted during the experimental process that large piles of cells were easier to find in the Ca^{2+} hydrogels than in the SCL hydrogels. We attribute this to cells in the Ca^{2+} hydrogels being able to survive and regenerate, due to proper growth signals secreted from adjacent groups of cells; cells in Ca^{2+} hydrogels can build connections with each other more easily, due to the shorter distance between them.
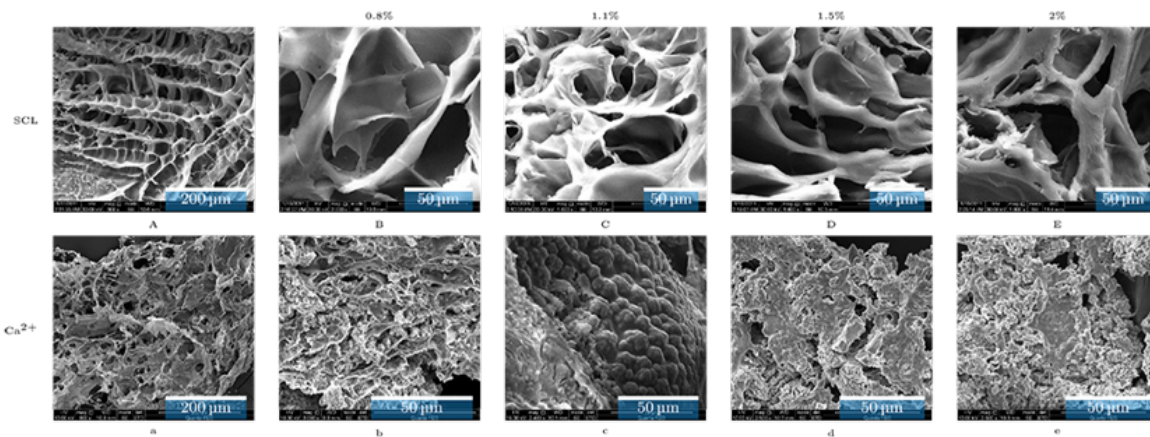
Figure 3.1: SEM images illustrating the morphology of SCL and Ca^{2+} hydrogels: Without cells (A for SCL hydrogel and a for Ca^{2+} hydrogels) and with cells (B–E for SCL hydrogel and b–e for Ca^{2+} hydrogels, both with varying concentrations of 0.8%, 1.1%, 1.5%, and 2%, respectively).

3.4.2   Mechanical Strength

Four trendlines indicating the mechanical strength of the SCL hydrogels and Ca^{2+} hydrogels are shown in Figure 3.2 A,B, respectively. Figure 3.2 A illustrates that the 2% SCL hydrogel had the highest mechanical strength (0.57×10^4 Pa), followed by slightly lower strength in the 1.5% SCL hydrogel (0.53×10^4 Pa), the considerably lower strength in the 1.1% SCL hydrogel (0.28×10^4

Pa), and the lowest strength in the 0.8% SCL hydrogel (0.76×10^3 Pa). Figure 3.2 B shows that Ca^{2+} hydrogels follow a similar trend in mechanical strength; the 2% Ca^{2+} hydrogel had the highest strength (0.23×10^5 Pa), followed by the 1.5%, 1.1%, and 0.8% Ca^{2+} hydrogels (at 0.216×10^5, 0.14×10^5, and 0.68×10^4 Pa, respectively). The mechanical strength results indicate that Ca^{2+} hydrogels are stronger than SCL hydrogels; the weakest Ca^{2+} hydrogel (0.8%) had a similar strength to the strongest SCL hydrogel (2%).
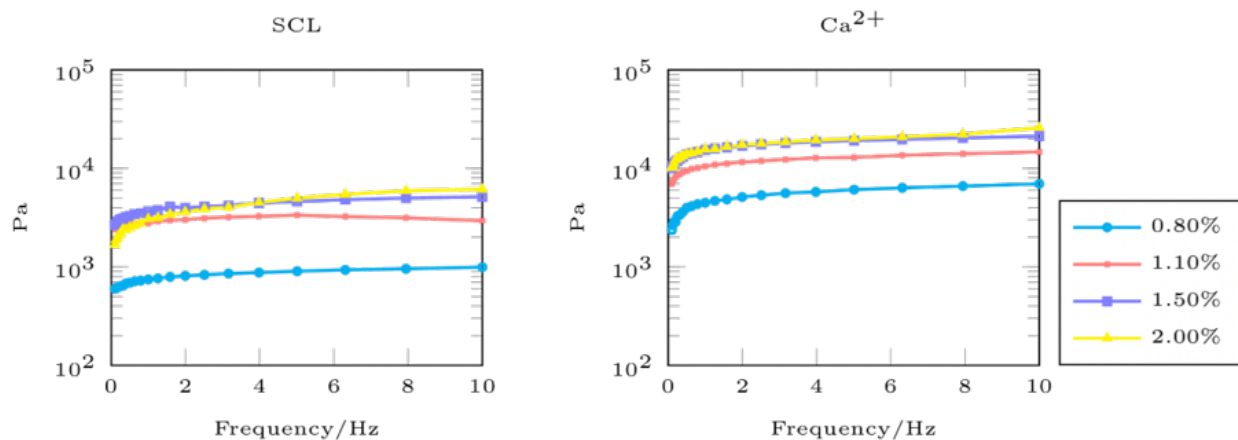
Figure 3.2: Rheometry data with respect to the mechanical strength of the hydrogels. 0.8%, 1.1%, 1.5%, and 2% are the concentrations of alginate in both types of hydrogels, and the varying concentrations of alginate present for the different groups of hydrogels. (A) The mechanical strength of SCL hydrogels with changing frequencies; (B) The mechanical strength of Ca^{2+} hydrogels with changing frequencies.

3.4.3   Degradation

The rate at which the hydrogels degraded (in terms of weight change) in the mimic in vivo environment, is shown in Figure 3.3 A, B. The degradation of the SCL hydrogel groups varied (Figure 3.3 A). Specifically, in the first 96 h, the 0.8% SCL hydrogel degraded the fastest, the 2% SCL hydrogel degraded the slowest, and the 1.1% and 1.5% SCL hydrogels had similar degradation rates, that were slightly slower than the 2% SCL hydrogel. Thereafter, the hydrogels in all four groups disappeared, indicating complete degradation. In contrast, all four Ca^{2+} hydrogels demonstrated similar trends and degradation rates in the presence of the enzyme (collagenase type II), until they disappeared completely. This indicates that their degradation is solely a function of time and is not affected by the concentration of alginate.

The varying degradation rates across the SCL hydrogel groups is likely due to their different mechanical strengths. The hydrogels with the lowest mechanical strength degraded the fastest, whereas the hydrogels with a higher mechanical strength degraded more slowly. In contrast, all Ca^{2+} hydrogels degraded more slowly than the SCL hydrogels, due to their collectively higher mechanical strengths. This suggests that all of the Ca^{2+} hydrogels were strong enough to resist the action of the enzyme, as reflected in the rather slow rate of degradation; thus, the only factor affecting the degradation rate was time. The 2% SCL hydrogels, the strongest of the SCL hydrogels, demonstrated the slowest degradation rate, and only a slight weight loss in the first 48 h after exposure to the enzyme.
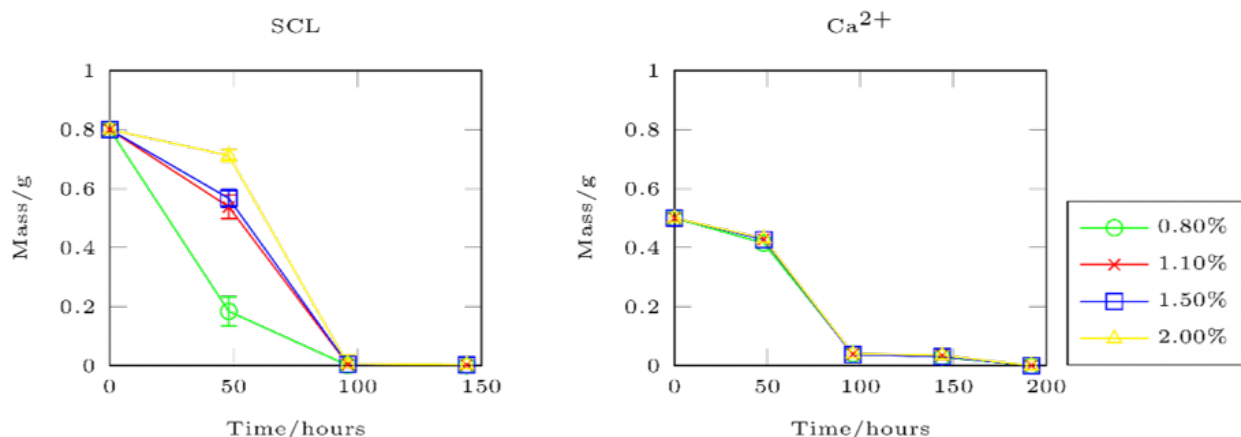
Figure 3.3: Degradation profiles of SCL and Ca^{2+} hydrogels. 0.8%, 1.1%, 1.5%, and 2% are the concentrations of alginate in both types of hydrogels, and the varying concentrations of alginate present for the different groups of hydrogels. (A) The mass change of SCL hydrogels with hours. (B) The mass change of Ca^{2+} hydrogels with hours.

3.4.4 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Staining

The percentage of living cells at 0 h, 2 h, 24 h, and two months after encapsulation in the hydrogels are presented in Figure 3.4. Specifically, the cell viability right after the injection process was measured and set as the baseline for the comparison with those measured at different time periods, which is shown as a percentage thereof.

For the SCL hydrogels, data from 2 h after encapsulation show that cell viability decreases with an increasing alginate concentration. Specifically, the 2% SCL hydrogels had the lowest cell

viability and the 0.8% SCL hydrogels had the highest. This suggests that cell viability during the injection process varies with hydrogel viscosity, with more cells surviving in the lower viscosity hydrogels. After 24 h, the percentage of living cells in all of the groups increased, compared to those at 0 h, particularly the 1.5% and 2% SCL hydrogels (t-test, $p<0.01$), which had a higher cell viability than the other two groups. The increased number of living cells indicates that cells proliferated after being encapsulated in the hydrogels, and the low cell viability in lower viscosity hydrogels indicates that the cells survived better in the higher viscosity hydrogels. After two months, cell numbers decreased compared to those at 2 h and 24 h, and 0.8% SCL hydrogels had the lowest living cells rate out of all of the groups (t-test, $p<0.05$). This indicates that living cell rates decreased with the degradation of hydrogels, due to a lack of protection.

For the Ca^{2+} hydrogels, cell viability, assessed by MTT assay, was not significantly different across the four groups after 2 h (t-test), and all viability rates were less than those for the SCL hydrogels (t-test, $p<0.05$). This indicates a lower retention rate of living cells in Ca^{2+} hydrogels, which have a higher viscosity than the SCL hydrogels, during the cell encapsulation process. After 24 h, the cell viability sharply increased compared to the baseline, especially for the 1.1% Ca^{2+} hydrogel, which had the highest cell viability (t-test, $p<0.01$). As such, cells seem to prefer hydrogels with a higher mechanical strength, in order to survive and proliferate. After two months, cell viability rates in the Ca^{2+} hydrogels still demonstrated an increasing trend, when compared to both the baseline and 24 h values. In particular, the 1.1% Ca^{2+} hydrogel had the highest living cell rate among the four groups. This suggests that cells were effectively regenerated during extended cultivation in hydrogels and proliferated the most in the high mechanical strength 1.1% Ca^{2+} hydrogels. After two months, the living cell rates for all Ca^{2+} hydrogels were much higher than those for SCL hydrogels. This reflects the fact that Ca^{2+} hydrogels have a slower degradation process than SCL hydrogels and that cells are still protected in the hydrogels after two months. We speculate that, during these two months, the cells proliferated and interconnected to each other, forming a network structure similar to the ECM that provides protection for cells in in vivo conditions.

The mechanical properties of hydrogels are important factors affecting the cell viability, as discussed above. It should be noted that, in addition to the mechanical properties, many other factors, such as the presence of residual reagents, pore density, and apoptotic potential of gelatin fragments, may also contribute to the cell viability.
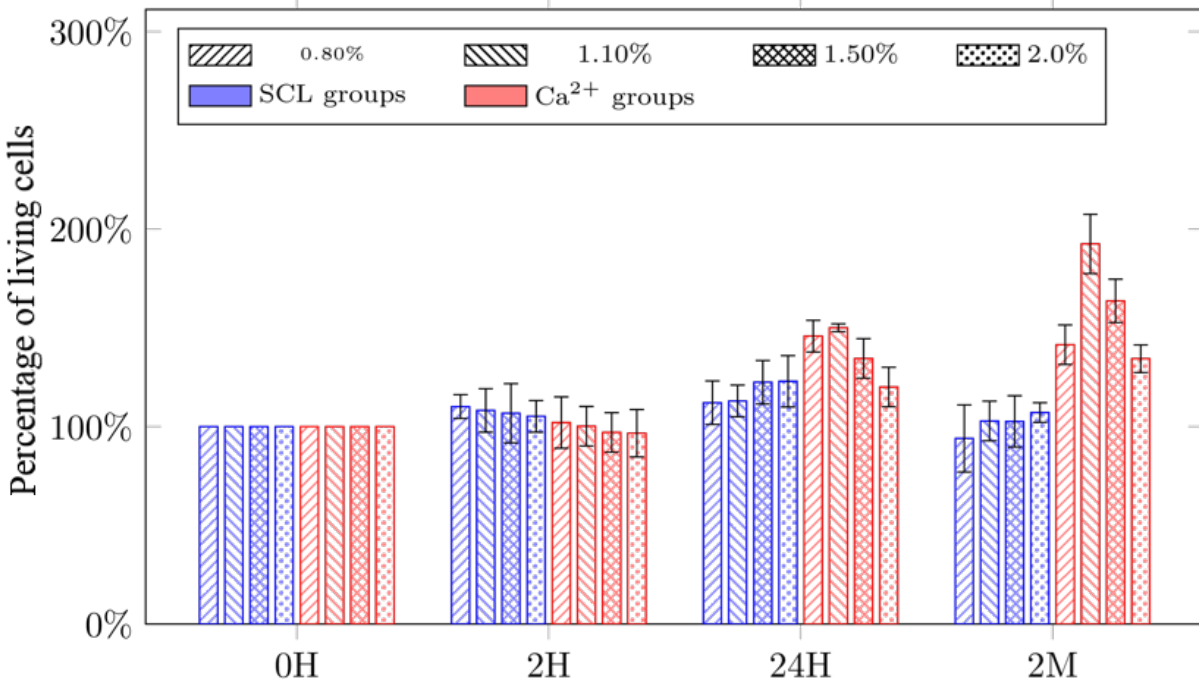
Figure 3.4: The percentage of living cells in SCL and Ca^{2+} hydrogels at different time intervals measured by absorbance of optical density at 595 nm using MTT assay. 0.8%, 1.1%, 1.5%, and 2% are the concentrations of alginate in both types of hydrogels. t-test was used to test the statistical significance.

3.4.5 Fluorescent Staining

The cells were stained with PI (red)/calcein (green)/DAPI (blue) to indicate dead cells/live cells/cell nuclei. The images were captured via confocal microscopy and are shown in Figure 3.5, for only one layer of cells in the hydrogels. There are staining in other adjacent layers got mixed to the current layer despite lower concentrations of stains being applied to minimize misleading color from adjacent layers during measurement. The proportion of the number of green cells to the number of red cell in the SCL hydrogels was, in general, less than in the Ca^{2+} hydrogels, which indicates that the cell survival rate in the Ca^{2+} hydrogels is higher than in the SCL hydrogels.

3.5 Limitations and Future Work

Cell differentiation from MDSCs into cardiomyocytes is important for MI repair. In this work, cell differentiation from MDSCs into cardiomyocytes was not experimentally tested, as we only
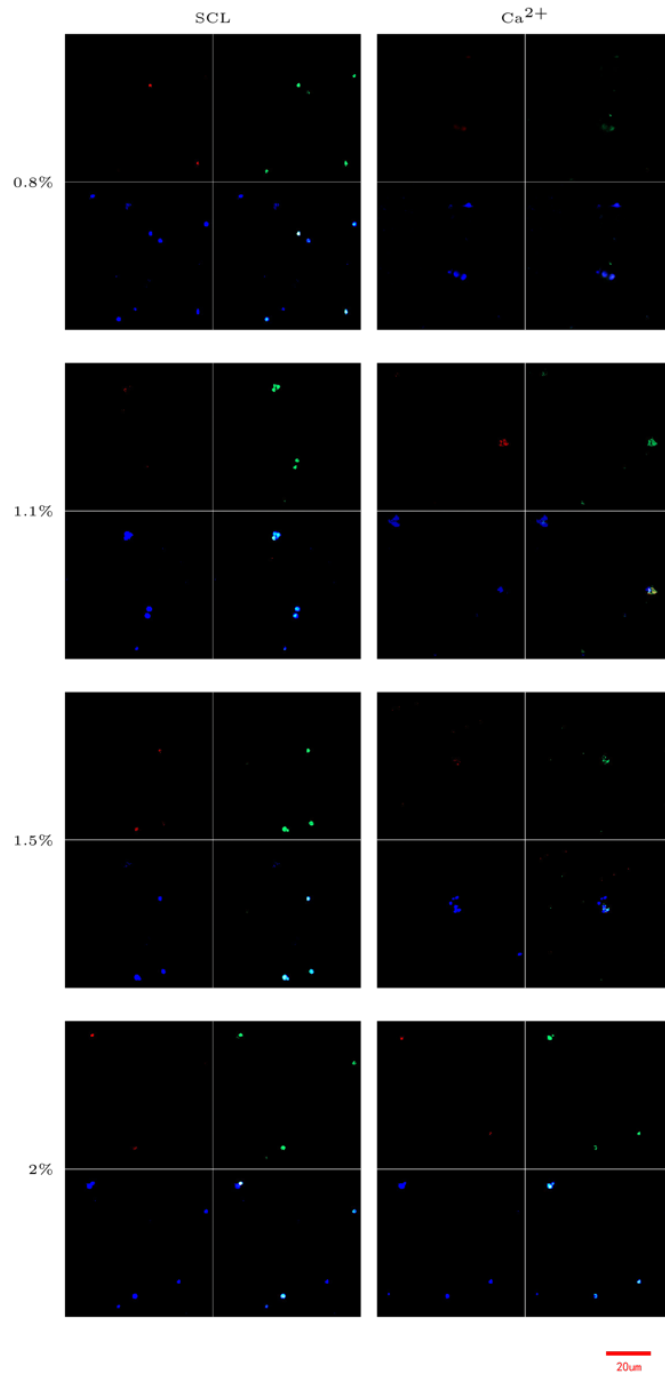
Figure 3.5: Fluorescent staining of live/dead cells in SCL and Ca^{2+} hydrogels. 0.8%, 1.1%, 1.5%, and 2% are the concentrations of alginate in both types of hydrogels, and the varying concentrations of alginate present for the different groups of hydrogels.

focused on optimizing the cell survival rate by adjusting the hydrogel formulation. In future work, we will study how MDSCs differentiate into cardiomyocytes and compensate for diminished viable cell numbers in MI hearts. Different stimulations inducing cell differentiation will be analyzed and the beating ability of differentiated cells will also be evaluated.

3.6   Conclusions

Injecting hydrogel-encapsulated cells shows promise for compensating for the loss of cardiomyocytes in MI repair. In this paper, we present a study on two types of alginate hydrogels, self-crosslinked and calcium-ion cross-linked hydrogels (or SCL and Ca^{2+} hydrogels), to encapsulate muscle-derived stem cells for potential MI repair. Our results illustrate that the cells can survive and proliferate in both hydrogels over the examined time period, from their injection to two-month cultivation. Our results also illustrate that cells have a better viability in SCL hydrogels than Ca^{2+} hydrogels right after injection and for the first 24 h after encapsulation. However, the cell survival rate is much higher in Ca^{2+} hydrogels than in SCL hydrogels after long-term (two month) cultivation. Our efforts to select the best formation of hydrogel for optimizing cell survival, show that SCL hydrogels with 0.8% alginate and 20% gelatin achieve the best cell viability during the injection process. However, Ca^{2+} hydrogels with 1.1% alginate and 20% gelatin have the highest cell survival rate during long-term cultivation, which is promising for the high-level regeneration of cells for potential MI repair.

4 Conclusions and Future Work

4.1 Summaries and Conclusions

In this study, two tissue-engineering strategies, as controlled release of BIO + IGF-1 via pH sensitive hydrogel system and encapsulating living cells with alginate hydrogel for repairing MI were developed and studied in Chapter 2 and Chapter 3, respectively. Experiments were designed and performed in accordance to these strategies. Based on experimental results obtained, main conclusions can be drawn as below.

4.2 Conclusions from Chapter 2

Chapter 2 presents the study on the sustained release of BIO and IGF-1 in a pH sensitive hydrogel simulating endogenous cardiac repair in MI rats. Gelatin nanoparticles encapsulating BIO and/or IGF-1 were fabricated successfully. In this novel hybrid hydrogel system, the gelatin nanoparticles were efficiently cross-linked with the oxidized alginate, which was developed to deliver the grow factors specifically to the MI site. Then the characteristics of nanoparticles and hydrogel system were investigated and described. The controlled release profile of the growth factors was described. From the in vivo results, the hybrid hydrogel system can enhance the proliferation of resident cardiac cells as well as promoting revascularization around the MI sites, allowing improved cardiac function and resulting in enhanced heart function recovery. These findings indicate that the hybrid hydrogel system can co-deliver BIO and IGF-1 to MI areas and thus repair cardiac function by promoting the regeneration of cardiomyocytes and revascularization. And this novel hybrid hydrogel delivery system is holding promise for treating MI and other related diseases requiring specific concentrations of drugs given the ability of delivery a wide range of therapeutics.

4.3   Conclusions from Chapter 3

Chapter 3 presents the study of encapsulating living cells in injectable alginate hydrogel for potential MI animal repair. Two types of alginate hydrogels as self-crosslinking (SCL) and calcium ion crosslinking (Ca^{2+}) with varying formulations were fabricated successfully. Living muscle-derived stem cells (MDSCs) were encapsulated in both types of hydrogels, and the experimental results illustrate that these living cells were able to survive and proliferate from the moment of encapsulating and until two months after encapsulation in both hydrogels. The compositions of both hydrogels were selected by evaluating the encapsulated living cells performance for further optimizing cell viability during the injection process and the live/dead cell rate in long-term cultivation. The characterization of both types of hydrogels was described, the morphology was characterized by scanning electronic microscopy (SEM) and the mechanical properties were examined via a rheometer. The experimental results of examined cell survival rate demonstrate that cells have a better viability in SCL hydrogels than Ca^{2+} hydrogels right after injection and for the first 24 h after encapsulation, especially the SCL hydrogel with a 0.8% alginate and 20% gelatin formulation which had the highest cell viability during the injection process. However, the cell survival rate is much higher in Ca^{2+} hydrogels than in SCL hydrogels after long-term cultivation, particularly, the Ca^{2+} hydrogel composed of 1.1% alginate and 20% gelatin maintained the highest cell survival rate after two months in culture. The cell survival rate after long-term cultivation shows promise for the high-level regeneration of cells for potential MI repair.

4.4   Future Work

In this thesis, two strategies were developed based on injectable hydrogel, i.e., 1) combining with nanoparticles for controlled release of growth factors and 2) encapsulating living cells for MI repair had been studied. The experimental results obtained from both strategies which shows in chapter 2 and 3 are promising in MI repair.

It is noted that the research of strategy 1) was performed both in vitro and in vivo and that the development of strategy 2) was pursued only in vitro though results showed promise for potential MI repair. While the research in MI animal models is yet to be studied for strategy 2). Thus, for

future work, the in vivo experiments of injectable hydrogel encapsulating living muscle derived stem cells shall be performed. The research objectives should be to study the living cells' behavior and how this strategy works in animal's hearts.

Specifically, the first step is to investigate whether the transplanted muscle derived stem cells can undergo differentiation into other types of cells and what would be the conditions for potential differentiation in vivo. From literature review, muscle derived stem cells are able to differentiate into cardiomyoycytes with proper stimulations in vitro. But there is rare reference showing these stem cells can differentiate into cardiomyocytes in animals' MI repair. Hence, to investigate the stimulations as specific conditions for MDSCs for potential differentiation is highly recommended. There are two main proposed clues as physically stimulation and chemical drugs/growth factors for stimulating MDSCs differentiating into cardiomyocytes. In details, to investigate whether simply physical stimulation of adding pressures on these cells can stimulate them differentiation. If these cells were able to differentiate under physical stimulation, then the load range that can cause the differentiation could be studied. The other possible clue to study the differentiation is proper chemical molecules. For example, the specific family of growth factors should be chosen from huge numbers of growth factors and the proper concentration of these chosen growth factors should be determined.

From my preliminary experiments results on the differentiation of 3D cultivated muscle-derived stem cells with growth factor TGF-b of the concentration at 2.5ng/ml and 5 ng/ml, it showed there were cells stimulated into cardiomyocytes since the cardiac specific protein TnT after 14 days cultivation with TGF-b at 5 ng/ml was examined. For future work, researchers may continue investigate the specific concentration of TGF-b for muscle-derived stem cells differentiation and the appropriate cultivation time of the growth factor with cells.

The second step is to study the in vivo tests. Although 3D cultured cells with proper stimulation for cells to differentiate into cardiomyocytes is the mimic of the in vivo, the real environment in body can make huge differences due to the existence of various enzymes and their kinetic profile. Thus to investigate whether muscle-derived stem cells can be differentiate into cardiomyoyctes is important. Briefly, the study can be performed in MI animal hearts by transplanting MDSCs with growth factors for some weeks and then examine whether there is the expression of cardiac tissue specific antibody. In general, this study can start with the cultivation of GFP-labeled (green fluo-

rescent protein) muscle-derived stem cells with growth factors for a few days until the expression of specific protein can be examined. Then the growth factors could be released in a controlled manner via nanoparticles to maintain their specific functioning concentration. And these differentiated cells with the nanoparticles encapsulated growth factors to be transplanted into MI animals' hearts for few days then examine the heart of these transplanted GFP cells to see whether there is the expression of cardiac specific protein.

The last step is to investigate the integrated effects of hydrogel with living cells in MI animals. The enhanced function of MI hearts can be achieved through either proliferated cardiomyocytes and differentiated cardiomyocytes, or the angiogenesis of the small arteries which can provid blood-efficient environment for cells to survive. Thus, to examine whether the combination of living cells combining hydrogels with growth factors works in regeneration ability of cardiomyocytes and the revascularization ability after transplanting into MI animal hearts is important. The combining system of hydrogel with living cells and growth factors should be fabricated and examined before transplanting into MI animals' hearts.

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