**A VEGF delivery system targeting MI improves angiogenesis and cardiac
function based on the tropism of MSCs and layer-by-layer self-assembly**

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Abstract: Myocardial infarction (MI) is a serious ischemic condition affecting many individuals around the world. Vascular endothelial growth factor (VEGF) is considered a promising factor for enhancing cardiac function by promoting angiogenesis. However, the lack of a suitable method of VEGF delivery to the MI area is a serious challenge. In this study, we screened a suitable delivery carrier with favorable biocompatibility that targeted the MI area using the strategy of an inherent structure derived from the body and that was based on characteristics of the MI. Mesenchymal stem cells (MSCs) are important infiltrating cells that are derived from blood and have an inherent tropism for the MI zone. We hypothesized that VEGF-encapsulated MSCs targeting MI tissue could improve cardiac function by angiogenesis based on the tropism of the MSCs to the MI area. We first developed VEGF-encapsulated MSCs using self-assembled gelatin and alginate polyelectrolytes to improve angiogenesis and cardiac function. In vitro, the results showed that VEGF-encapsulated MSCs had a sustained release of VEGF and tropism to SDF-1. In vivo, VEGF-encapsulated MSCs migrated to the MI area, enhanced cardiac function, perfused the infarcted area and promoted angiogenesis. These preclinical findings suggest that VEGF-loaded layer-by-layer self-assembled encapsulated MSCs may be a promising and minimally invasive therapy for treating MI. Furthermore, other drugs loaded to layer-by-layer self-assembled encapsulated MSCs may be promising therapies for treating other diseases.

Keywords: myocardial infarction; targeting VEGF-encapsulated MSCs; layer-by-layer self-assembly; tropism to the MI zone.

Introduction

Myocardial infarction (MI) is a leading cause of death and creates a significant burden on patients. No effective method has been developed to protect against myocardial ischemia [1]. MI is characterized by a loss of blood flow to the affected region of the heart, and the prognosis is related to angiogenesis and the length of time of artery obstruction [2]. In recent years, improving angiogenesis in the ischemic myocardium has become the focus of MI research [3].

Vascular endothelial growth factor (VEGF) is the most important factor in angiogenesis [4]. VEGF has been demonstrated to stimulate endothelial cell proliferation, promoting angiogenesis by binding to high-affinity receptors, including the tyrosine kinase receptor Flk-1 and the fms-like tyrosine kinase receptor Flt-1, and it is expressed predominantly on endothelial cells [5, 6]. Intravenous administration of VEGF has shown a slight effect on the reconstruction of cardiac microvasculature and enhanced cardiac function, partly because of the short half-life (approximately several minutes) of VEGF in the blood [4, 7]. Increasing the dose of VEGF may increase its availability to the target tissue; however, an increase in the risk of the development of serious conditions such as neoplasms, diabetic retinopathy, rheumatoid arthritis, and atherosclerosis may occur [8-11]. Targeting the delivery of an adequate therapeutic dose of VEGF to the heart is a key challenge.

1 The targeted delivery of an adequate therapeutic dose of VEGF to the infarcted
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3 zone is very important [12-15], as it could promote the formation of new vessels in
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5 the infarcted zone while reducing the risk of potential side effects in other tissues. In
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7 recent studies, Kiani and his colleagues reported that targeting VEGF-encapsulated
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9 immunoliposomes to the MI area of the heart improved vascularity and cardiac
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11 function [16]. A previous study showed that CBD-VEGF could improve cardiac
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13 performance after myocardial infarction via targeting collagen in the heart [17].
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15 However, the poor biocompatibility and inconvenience of the delivery system were
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17 great challenges [6].
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25 In this study, we screened a suitable delivery carrier with good biocompatibility
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27 that targeted the MI area using the strategy of screening an inherent structure derived
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29 from the body based on characteristics of the MI. Acute myocardial infarction causes
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31 sterile inflammation, which is characterized by recruitment of MSCs to the area of the
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33 MI [18]. The SDF-1/CXCR4 axis plays a vital role in chemotaxis, homing, and
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35 engraftment of MSCs in infarcted hearts [18-20]. SDF-1 is secreted by cells within the
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37 ischemic myocardium and controls MSCs recruitment [21, 22]. MSCs migrate toward
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39 infarcted myocardial tissue along an SDF-1 concentration gradient by binding SDF-1
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41 to CXCR4 on the surface of the MSCs [23]. As MSCs are derived from the blood,
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43 they have good biocompatibility.
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53 In this study, we hypothesized that the use of VEGF-encapsulated MSCs is a
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55 promising strategy to improve angiogenesis and cardiac function. The selection of a
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1 suitable technique to load VEGF onto MSCs that offers good biocompatibility and
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3 convenience is needed.
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6 Layer-by-layer (LbL) self-assembly is a thin-film fabrication technique of
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8 coating cells that works by depositing alternating layers of oppositely charged
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10 biocompatible materials (polycations and polyanions) on the cell surface. This
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12 technique has been studied for encapsulating fungi [24], bacteria [25] and mammalian
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14 cells, and it maintains the metabolic activity of the encapsulated material and endows
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16 protection [26]. Jiming Kong and his colleagues reported that LbL encapsulation with
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18 gelatin and alginate polyelectrolytes does not affect the viability, proliferation, or
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20 differentiation of cells [27]. The LbL self-assembly technique has reportedly been
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22 used for drug delivery [28, 29]; for example, insulin [30], doxorubicin [31], and bone
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24 morphogenetic protein-2 (BMP-2) have been loaded onto an LbL structure, showing
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26 excellent sustained release.
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36 The aim of this study was to determine whether VEGF-encapsulated MSCs by
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38 layer-by-layer self-assembly with polyelectrolytes is a good targeting vehicle to
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40 improve angiogenesis and cardiac function. When VEGF-encapsulated MSCs were
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42 injected into the body, homing of the VEGF-encapsulated MSCs to the MI tissue
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44 occurred, and VEGF was released to enhance angiogenesis and cardiac function
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46 (Graphical Abstracts). In this study, we first developed a new targeting carrier for
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48 MSCs with a VEGF-loaded gelatin and alginate polyelectrolyte assembly. The results
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50 suggest that VEGF-encapsulated MSCs via a loaded polyelectrolyte layer-by-layer
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52 porous assembly may be a promising, minimally invasive therapy for treating MI.
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2. Materials and methods

2.1 Cell culture

Rat mesenchymal stem cells (MSCs) were isolated from the bone marrow of sacrificed Sprague-Dawley (SD) rats and cultured using the whole bone marrow adherence method with Mesenchymal Stem Cell Growth Medium (rMSC-GM) (Cyagen, Guangzhou, China). Cells were in an undifferentiated state within five passages. The cells from the third passage were used in the experiments for this study. MSCs were cultured in rMSC-GM containing 10% FBS (Hyclone, Utah, USA) and antibiotics in an incubator containing 95% air and 5% CO₂.

2.2 Preparation and characterization of LbL-MSCs

2.2.1 Construction of LbL-MSCs Recombinant human VEGF 165 protein (VEGF) was purchased from R&D Systems, USA. The VEGF-encapsulated MSCs were assembled using the layer-by-layer technique with gelatin and alginate (Sangon Biotech, China), as previously reported [24, 32] and shown in Figure S1 (Supporting Information). The experimental detail was shown in experimental section (Supporting Information). Briefly, first, 5×10^6 MSCs were centrifuged in a 15-mL centrifuge tube to remove the medium. Next, 5 mL of a 0.05% gelatin solution (10 µg VEGF was mixed with gelatin to allow for adsorption) was added to the tube, and the tube was gently shaken (5 min, 70 r/min). After the cells were washed by Dulbecco's phosphate-buffered saline (DPBS), 5 mL of 0.05% alginate was incubated with the cells, and the tube was gently shaken (5 min, 70 r/min). Then, cells were washed by

1 DPBS, 5 mL of 0.05% gelatin solution (10 μ g VEGF was mixed with gelatin to allow
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3 for adsorption) was added to the tube, and the tube was gently shaken (5 min,
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5 70r/min). Then, cells were washed by DPBS, the three-layered LbL encapsulation
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7 model of MSCs was accomplished. Control-MSCs with gelatin and alginate
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9 layer-by-layer assembly were prepared as described above, but VEGF was not loaded.
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12 LbL-ECs (LbL-endothelial cells) were prepared according to the same procedure as
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15 LbL-MSCs.
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20 Cells were divided into the following 3 groups: MSCs group (No treat),
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22 LbL-MSCs group (VEGF-loaded gelatin and alginate polyelectrolyte encapsulated)
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24 and Control-MSCs group (gelatin and alginate polyelectrolyte encapsulated).
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28 **2.2.2 TEM of LbL-MSCs** The LbL-MSCs were processed as previously
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30 reported and prepared for viewing using transmission electron microscopy (TEM)
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32 [27]. MSCs and LbL-MSCs were fixed in a solution of 2.5% glutaraldehyde (diluted
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34 with 0.1 M Sorensen's buffer) for 1 h. The samples were then washed three times
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36 with 0.1 M Sorensen's buffer) for 1 h. The samples were then washed three times
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38 with 5% sucrose solution (diluted with 0.1 M Sorensen's buffer) for 5 min each time
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40 and stored overnight in a refrigerator at 4°C. The samples were post-fixed by adding
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42 1% O_3O_4 to the cell samples for 2 h and dehydrated with graded alcohols and
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44 methanol, after which they were embedded in epoxy resin. A microtome (318423,
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46 Reichert Nr.) was used to obtain thin sections, and these sections were mounted on
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48 copper grids. Finally, the samples were stained with 2% uranyl acetate and 1% lead
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50 citrate. The LbL-MSCs were analyzed using a TEM (CM-10, Philips Netherlands).
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1 The TEM analysis was completed by the Biomedical Analysis Center of the Third
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3 Military Medical University.
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6 **2.2.3 The presence of VEGF** The presence of VEGF on the surface of the
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8 LbL-MSCs was revealed using anti-VEGF (Green, Abcam, USA) and fluorescence
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10 microscopy. Briefly, the MSCs, LbL-MSCs and Control-MSCs were fixed in 4% PFA
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12 at 4°C on glass substrates overnight. They were then washed three times (5 min each)
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14 with 0.1 MPBS. Nonspecific binding on MSCs was blocked by 1% BSA in TBST for
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16 30 min. Next, the cells were incubated with anti-VEGF (diluted 1:50 using PBS), and
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18 samples were washed three times (5 min each) with DPBS. Images were taken in 10
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20 random fields using a fluorescence microscope (TE2000-E, Nikon, Japan). Positive
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22 cells were quantified by IPP6. 0.
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31 **2.2.4 The zeta-potential of LbL-MSCs** The zeta-potential of the LbL-MSCs
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33 and MSCs was determined using a zeta-potential analyzer (Brookhaven, USA).
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35 Briefly, 2×10^6 MSCs and LbL-MSCs (encapsulated with gelatin, gelatin/alginate, and
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37 (gelatin)₂/alginate) were analyzed after preparation of the LbL-MSCs (encapsulated
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39 with gelatin, gelatin/alginate, or (gelatin)₂/ alginate).
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45 **2.2.5 SEM of LbL-MSCs** The MSCs and LbL-MSCs were analyzed using
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47 scanning electron microscopy (SEM) for time points at 1, 3 and 7 days. The MSCs
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49 and LbL-MSCs were fixed in 0.1 M Sorensen's buffer (containing 2.5%
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51 glutaraldehyde) for 2 h. The samples were washed three times (5 min each) with 0.1
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53 M Sorensen's buffer (containing 5% sucrose). Next, 1% O_sO₄ was added to the
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55 samples for 4 h at 4°C, followed by three cycles of washing. After being dehydrated
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1 with graded alcohols and dried, the samples were sprayed with gold and viewed using
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3 SEM (JEOL 5900). The SEM analysis was completed by the Biomedical Analysis
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6 Center of the Third Military Medical University.
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9 **2.2.6 Cell viability test with Hoechst/PI Staining** LbL-MSCs, Control-MSCs,
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11 MSCs and MSCs treated VEGF (20 ng/mL) were fixed in a solution of 4%
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13 paraformaldehyde (PFA) in DPBS at 4°C for 15 min. Enough solution was used to
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15 cover dish thoroughly. The fixing solution was aspirated, and cold DPBS was added
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17 to thoroughly rinse the solution three times. A 1:1000 dilution of Hoechst 33258 stock
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19 (Sigma, USA) in DPBS (diluted fresh each time; final concentration was 0.12 µg/mL)
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21 was added. The solution was incubated in the dark for 15 min at room temperature.
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23 Then, it was rinsed approximately five times with DPBS. Then, a 1:1000 dilution of
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25 PI (final concentration was 0.3 µg/mL) was added. The solution was again incubated
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27 in the dark for 3 min at room temperature. Finally, the samples were observed with a
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29 fluorescence microscope (TE2000-E, Nikon), from which images of ten random fields
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31 were taken for counting.
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42 **2.2.7 Evaluation of cell differentiation using RT-PCR** It is well known that
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44 MSCs differentiation potential occurs according to three lineages, osteogenic,
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46 chondrogenic and adipogenic. LbL-MSCs, Control-MSCs, and MSCs differentiation
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48 potentials were examined through gene expression with RT-PCR. The RT-PCR
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50 experimental method and primers are shown in the experimental section (Supporting
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52 Information).
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2.2.8 Persistence of coated materials Gelatin-rhodamine B is prepared to show coated materials as described in the experimental section (Supporting Information). First, we encapsulated 5×10^6 MSCs with gelatin-rhodamine B and ordinary alginate, as described above. Then, we moved 1×10^4 LbL-MSCs to a 96-well plate, and the solutions were diluted to 100 μ L with DPBS. Next, we examined the fluorescence intensity using a multilabel counter (1420, Wallac) at an excitation wavelength of 590 nm over a dynamic period.

2.3 Determination of the tropism of MSCs in vivo and in vitro

There is a need to determine whether a single MSCs-based LbL encapsulation with gelatin and alginate can influence the tropism of MSCs.

2.3.1 SDF-1/CXCR4 binding on the surface of LbL-MSCs In a previous study, MSCs migrated toward the infarct zone along an SDF-1 concentration gradient by binding SDF-1 to CXCR4 on the surface of the MSCs [23]. In this study, the binding of SDF-1/CXCR4 on the surface of LbL-MSCs (cultured at different times) was determined with SDF-1 (labeled red with rhodamine B, Abcam, USA, Supporting Information) using fluorescence microscopy. Briefly, cells were grafted on coverslips were fixed in 4% PFA for 15 min at 4°C. They were then washed three times (5 min each) with 0.1 M PBS. Nonspecific binding on MSCs was blocked by 1% BSA in TBST for 30 min. Next, the cells were incubated with SDF-1 (labeled red 1:50), and samples were washed three times (5 min each) with PBS. Images were taken in 10 random fields using a fluorescence microscope (TE2000-E, Nikon). Positive cells were quantified by IPP6.0 (repeated three times).

2.3.2 Tropism of LbL-MSCs using the co-culture system The tropism of LbL-MSCs and MSCs was determined in vitro using a Transwell 24-well co-culture system (pore size: 8 μ m, Corning Costar, USA). A total of 100 μ L of the LbL-MSCs and MSCs suspension (1×10^5) was added to the upper chambers, and 500 μ L of the chemokine solution (SDF-1, 1 ng/mL, BioVision, USA) was added to the lower chambers. For analyzing the tropism of LbL-MSCs towards SDF-1 in vitro, the co-culture system was incubated in a humidified chamber (37°C, 5% CO₂) for 36 h, and the cells that permeated the membrane and attached to the lower surface were then stained with crystal violet. The average number of cells was counted in five random 100 $\times$ fields under a microscope (repeated three times).

2.3.3 Scratch assays Scratch assays were established using previously published methods [33] in protein-coated tissue culture plates. Cells were stained with crystal violet, and cell migration was evaluated under a microscope (repeated three times).

2.3.4 Recruitment of LbL-MSCs in vivo The MSCs and LbL-MSCs were labeled with CellTracker™ Red (Red, Invitrogen, USA) according to the manufacturer's instructions and then encapsulated using layer-by-layer assembly as described previously. The LbL-MSCs and MSCs were cultured for 2, 7 days in an incubator containing 95% air and 5% CO₂, and then 2×10^7 MSCs were delivered to MI rats through a tail vein. After 2 days, SD rats were sacrificed to harvest the heart. The recruitment of MSCs was evaluated using a fluorescence microscope.

2.4 VEGF release in vitro

2.4.1 ELISA To determine VEGF release from the LbL-MSCs, Control-MSCs and MSCs at different times and pH values, LbL-MSCs, Control-MSCs and MSCs were cultured in an incubator containing 95% air and 5% CO₂, and the pH of another group medium was adjusted to 6.5 to mimic the acidic microenvironment of MI tissue. The conditioned medium was collected. The VEGF of the conditioned culture supernatants was determined using the ELISA method (R&D Systems, USA) according to the manufacturer's instructions, and the culture medium for the MSCs was used as the negative control. The paracrine effect of LbL thin films on LbL-MSCs was tested using ELISA.

The conditioned culture supernatants (LbL-MSCs, Control-MSCs and MSCs) were collected at 48 h. IL-1, TGF- β , HGF, IGF1 and AGPT1 of the LbL-MSCs, Control-MSCs and MSCs were tested with commercial kits according to the manufacturer's instructions.

2.4.2 RT-PCR RT-PCR was used to determine VEGF mRNA gene expression in LbL-MSCs, Control-MSCs and MSCs. The RT-PCR experimental method and primers are shown in the experimental section (Supporting Information).

2.4.3 Western blots Western blotting was performed to analyze the expression of VEGF within the infarct area using a procedure modified from a previous study [34]. The supernatant proteins were transferred to PVDF membranes using a wet transfer setup. Following the transfer, the membranes were blocked for 18 h at 4°C in TBS-T with 3% milk to prevent nonspecific antibody binding. The membranes were incubated in the following primary antibodies for 18 h in 3% milk at 4°C: (1)

1 polyclonal anti-mouse VEGF (Cell Signaling Technology) at a 1:1000 dilution and
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3 (2) monoclonal anti-mouse B-actin (Santa Cruz, Dallas, TX) at a 1:1000 dilution as a
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5 loading control. The blots were visualized using a LICOR Odyssey fluorescent
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7 imaging scanner. The density of protein expression was standardized to the loading
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9 control.
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13 **2.5 Rat MI model**

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17 This experiment involved the use of male SD rats weighing 250 to 300 g, which
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19 were provided by the animal center of the Third Military Medical University. All
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21 animal experiments in this study were approved by the Third Military Medical
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23 University Experimental Animal Ethics Committee to ensure reduction in suffering
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25 and full protection for the welfare of the animals.
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31 The MI model was prepared by modified reports [35, 36]. Briefly, male SD rats
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33 were anesthetized using chloral hydrate (10 g/kg) and atropine (1 mg/kg) by
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35 intraperitoneal injection and were fixed in a sterile console. The rats were intubated
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37 using a vein detained needle and mechanically ventilated using a rodent respirator
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39 (Harvard Rodent Ventilator Model 683). The parameter setup consisted of a
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41 respiratory rate of 60 breaths/min and a tidal volume of 3 mL/100 g body weight.
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43 Under sterile conditions, the muscle was bluntly dissected, the chest was opened using
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45 a thoracotomy at the point of an obvious heartbeat, and the pericardium was removed.
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48 The left coronary artery was blocked with 6-0 proline for 45 min, then removed to
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50 allow reperfusion, and intubation was removed after recovery of spontaneous
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52 breathing. The rats were housed in a laboratory animal room and allowed to take food
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1 and water freely. The animals were randomly assigned to three groups (LbL-MSCs
2 group, Control-MSCs group, and VEGF group, n=12 in each group). On days 2,
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6 2×10^7 LbL-MSCs were delivered to the MI rats via the tail vein as LbL-MSCs group,
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9 2×10^7 Control-MSCs and 10 ug VEGF were delivered to the MI rats via the tail vein
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11 as Control-MSCs group, and 10 μ g VEGF and 2×10^7 MSCs were delivered to rats via
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13 the tail vein as the VEGF group.
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16 17 **2.6 Assessment of vascular density and the perfused border zone of the heart**

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20 **2.6.1 Micro-CT** The perfused border zone of the heart was assessed using
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22 micro-CT at weeks 4. The rats were anesthetized with chloral hydrate (4%,
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24 intraperitoneal injection 1 mL/100 g), and iohexol (Tianheng Pharmaceutical
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26 Company, Nanoprobes, NingBo, China) was injected through the tail vein. The rats
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28 were placed in a supine position in the micro-CT scanner (Skyscan 1176, Bruker
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30 micro-CT, Kontich, Belgium, each n=9). The micro-CT was completed by the
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32 Biomedical Analysis Center of the Third Military Medical University.
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40 **2.6.2 Immunofluorescence** Animals were sacrificed at 2 days, weeks 1, 2, 4
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42 after MI through an overdose of anesthesia. The hearts were immediately excised and
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44 frozen in Tissue-Tek O.C.T. sections (8 μ m thick, n=10) that were taken from the
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46 infarct area. The survival and distribution of the MSCs were analyzed using
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48 fluorescence microscopy. Images were taken using a fluorescence microscope in a
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50 blinded manner (n=10). Angiogenesis was analyzed in the MI zone using CD31
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52 (CD31, 1:200, overnight, Santa Cruz Corp., USA) and donkey anti-mouse antibodies
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54 (1:800, Invitrogen Corp., Shanghai, China) to stain the samples for 1 h at room
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temperature. Images were taken using a fluorescence microscope in a blinded manner (n=8).

2.7 Assessment of cardiac function

Cardiac diastolic function was assessed using pulsed-wave tissue Doppler imaging at 3 days and weeks 1, 3, 6. After steady anesthesia (peritoneal injection, 10 g/kg chloral hydrate), the left ventricular diastolic dimension (LVDD) and left ventricular systolic dimension (LVSD) were measured using a 2-dimensional guided M-mode ultrasound system (Acuson Sequoia, Siemens Medical Solutions, Mountain View, California) and a 13 MHz linear transducer with a short-axis view of the level of the papillary muscles. Left ventricular percent fractional shortening (FS) and ejection fraction (EF) were calculated using the following equations: FS (%) was calculated as $[(LVDD - LVSD) / LVDD] \times 100\%$ and EF (%) was calculated as $(EDV - ESV) / EDV$. From an apical four-chamber view, the diastolic filling velocity (E-wave) and atrial filling velocity (A-wave) were measured across the mitral valve using pulsed-wave Doppler spectra. The E/A ratio represents diastolic function. All measurements were determined over three consecutive cardiac cycles.

2.8 Statistical analysis

The experimental data were analyzed by SPSS 18.0. Variance analyses were performed, and the results are expressed as the means and error of the means (EM). Each experiment was repeated at least three times. All p-values were two-tailed, and a $p < 0.05$ was considered significant.

3. Results

3.1 Encapsulated MSCs and their characterization in vitro

The VEGF-encapsulated MSCs were assembled using the layer-by-layer technique with gelatin and alginate, as shown in Figure S1 (Supporting Information). TEM demonstrated an LbL thin film coating on LbL-MSCs compared to untreated MSCs (Figure 1). The material layers on the encapsulated cells were approximately 5.8 ± 2.2 nm and were calculated using ImageJ. The zeta-potential of the LbL-MSCs was determined to obtain the change in the potential of cells with different layers of materials (Figure 2j). When the MSCs were coated with gelatin and alginate using the layer-by-layer self-assembly technique, the zeta-potential of the LbL-MSCs was changed (5 mV, -40 mV). The oppositely charged polyelectrolytes were bound together and overcompensated for each other.

Furthermore, anti-VEGF (green fluorescence) was incubated with LbL-MSCs to show the presence of VEGF on the surface of LbL-MSCs on day 0, 2, 7, which were coated with VEGF-loaded gelatin and alginate (Figure 2a, 2b, 2c). The Control-MSCs and MSCs did not show VEGF on their surfaces. This finding indicated the success of VEGF encapsulation.

The LbL-MSCs were analyzed using SEM on days 1, 3, and 7. The results showed that the attachment of LbL-MSCs was slower than MSCs, and LbL-MSCs showed a spheroid-like morphology on days 1, and 3. On day 7, the LbL-MSCs exhibited a spindle-like morphology, and MSCs exhibited spindle-like morphology on day 3. At 1, 3, and 7 days (Figure 3a), F-actin staining by phalloidin showed the effect

1 of encapsulation on the cytoskeleton of the LbL-MSCs compared to the MSCs. The
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3 cytoskeleton of the LbL-MSCs was much more restrained than the MSCs. The
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5 spreading area of each cell was calculated using ImageJ (Figures 3b, 3c).
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9 The persistence of the materials on the surface of LbL-MSCs was observed by
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11 encapsulating MSCs with gelatin-rhodamine B and alginate. Figure S2 (Supporting
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13 Information) shows the decrease in the fluorescence, which marked gelatin on the cell
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15 surface from 0 to 10 days. LbL thin films were gradually degraded and endocytosed
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17 by MSCs over a dynamic period, which is associated with gradually increased
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19 chemotaxis.
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25 We determined the cell viability of LbL-MSCs with Hoechst/PI staining, and
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27 the results are shown in Figure S4 (Supporting Information). The results reveal that
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29 LbL encapsulation did not significantly influence MSCs viability over a dynamic
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31 period. In short, gelatin and alginate are suitable for LbL encapsulation of MSCs,
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33 LbL-MSCs have better survival compared to MSCs and Control-MSCs [15].
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39 It is well known that MSCs differentiation potential occurs according to three
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41 lineages, osteogenic, chondrogenic and adipogenic. MSCs differentiation potentials
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43 were examined through gene expression with RT-PCR, and the results and primers
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45 are shown in Figure S5, (Supporting Information). The results show that there was no
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47 significant effect on differentiation of LbL-MSCs, Control-MSCs and MSCs.
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53 The paracrine effect of LbL thin films on LbL-MSCs was tested using ELISA,
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55 and the results show that IL-1, HGF, and IGF-1 were increased and TGF- β decreased
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1 in LbL-MSCs and Control-MSCs compared to MSCs, which is associated with
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3 hypoxia caused by LbL thin films (Figure S6, Supporting Information).
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6 **3.2 Recruitment of LbL-MSCs was slightly weakened in a time-dependent** 7 8 **manner in vitro and in vivo** 9

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11 Determination of the tropism of LbL-MSCs was a necessary first step. MSCs
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13 migrated toward MI tissue along an SDF-1 concentration gradient by binding SDF-1
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15 to CXCR4 on the MSCs [23]. We tested the ability of SDF-1 to penetrate the LbL thin
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17 films of the LbL-MSCs to bind to CXCR4 on the surface of the MSCs at different
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19 times, and the results are shown (Figure S3, Supporting Information). We found that
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21 the inhibition was decreased in a time-dependent manner. On day 0, no SDF-1
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23 penetrated the LbL thin films to bind to CXCR4 on the surface of the LbL-MSCs. On
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25 day 2, we found that some SDF-1 penetrated the LbL thin films of the LbL-MSCs to
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27 bind to CXCR4 on the surface of the MSCs. On day 7, SDF-1 (red) was incubated
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29 with the LbL-MSCs to show a red fluorescence both on the surface and cytoplasm of
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31 the LbL-MSCs, which indicated the success of SDF-1 binding to CXCR4 (Figure 4a).
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33 SDF-1 (red) was also incubated with LbL-ECs to show no red fluorescence on the
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35 surface of the LbL-ECs on day 7 as a negative control.
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47 The co-culture system of the Transwell 24-well experiments showed that the
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49 tropism of the LbL-MSCs was slightly weakened compared to untreated MSCs in a
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51 time-dependent manner. The tropism values of the MSCs were approximately 3 times
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53 greater than the tropism values of LbL-MSCs at 1 days and 1.5 times greater at 3 days
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(Figures 4b, 4c, 4d). On days 7 and 10, there was no significant difference in the tropism of LbL-MSCs to SDF-1 compared to MSCs.

In vitro scratch assays showed no significant difference in the migration of MSCs and LbL-MSCs at 1, 3, and 7 days. Additionally, the in vitro assays showed the attachment of the LbL-MSCs at 7 days. These results indicate that the LbL thin films of the LbL-MSCs had no influence on the migration of MSCs, and the weak tropism of the LbL-MSCs is not induced by migration (Figure 4d).

In vivo, the number of positive MSCs (red fluorescence) in the MI zone from the LbL-MSCs group, Control-MSCs group, and VEGF group were 5.0 ± 2.0 , 5.3 ± 2.3 and 12.7 ± 5.6 /HPF (high power field) on days 2, respectively. The MI-tropics of the LbL-MSCs and Control-MSCs groups were damaged by the self-assembled layer compared to untreated MSCs. Improvement in MI-tropics (9.6 ± 3.6 /HPF) was found with the LbL-MSCs group (cultured 7 days after encapsulation) compared to MSCs. This indicated that the MI-tropics of the LbL-MSCs group were enhanced over time (Figures 5a, 5b), which was similar to the findings in vitro.

3.3 The release of VEGF from LbL-MSCs in vitro

Western blotting was used to show the deposit of VEGF in the infarct area. The results showed that the LbL-MSCs group deposited the greatest amount of VEGF compared to the VEGF group and Control-MSCs group at different time points (Figure 6a). No significant difference was observed between the VEGF group and control-MSCs group.

We measured VEGF release from LbL-MSCs (VEGF-loaded) and Control-MSCs (non-VEGF-loaded LbL-MSCs) using ELISA and used the MSCs as the control group. The results show that Control-MSCs and LbL-MSCs tend to secrete VEGF compared to MSCs. However, more VEGF was detected in LbL-MSCs (VEGF-loaded) compared to Control-MSCs (103500 ± 25100 pg/mL vs 3521 ± 413 pg/mL) (Figure S7 Supporting Information). The total release of VEGF within 14 days was $6.15 \mu\text{g}$ for LbL-MSCs and $0.205 \mu\text{g}$ for Control-MSCs. Therefore, $5.995 \mu\text{g}$ VEGF was physically absorbed and loaded onto the gelatin coating of 5×10^6 MSCs.

Moreover, we measured VEGF using RT-PCR. The results show that LbL-MSCs (VEGF-loaded or not) have no differences. Furthermore, greater VEGF mRNA was expressed in LbL-MSCs (VEGF-loaded or not) compared to MSCs (Figure S7, Supporting Information).

The VEGF content released from the LbL-MSCs was calculated at different time points at a pH of 7.4. The results were $1.23 \text{ pg/cells (VEGF)}$ for the accumulated release within 14 days. The LbL-MSCs could achieve sustained released. Furthermore, we investigated the influence of the acidic microenvironment on VEGF release because a low pH is an important feature in the ischemic environment. The release profiles of LbL-MSCs at pH values of 7.4 and 6.5 were different (Figure 6b), exhibiting prolonged release at a pH of 6.5 compared to a pH of 7.4.

3.4 Improvement in vascular density and perfusion in the infarct area after MI

1 The in vivo micro-CT showed that the rate of perfusion of the myocardial
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3 border zone was significantly ($p<0.01$) increased in the LbL-MSCs group, with 70%
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5 of the border zone being perfused compared to the Control-MSCs group (49%) and
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7 the VEGF group (47%) at 4 weeks post-MI. LbL-MSCs therapy restored the
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9 perfusion of vessels to a level similar to that observed in normal cardiac tissue, as
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11 shown in Figure 7.
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17 Angiogenesis was shown using CD31 staining. LbL-MSCs therapy resulted in a
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19 significant increase in the vascular density of the myocardial border zone compared to
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21 the Control-MSCs group and VEGF group at 4 weeks post-MI (Figure 8). The
22
23 vascular density of the ischemic myocardium in the control-MSCs and LbL-MSCs
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25 groups was 3.6 ± 1.9 and 25.6 ± 5.4 (#/HPF), respectively. In addition, no significant
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27 difference in vascular density was observed between the groups before week 1
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29 post-MI ($p=0.15$). For the hearts treated with LbL-MSCs, the average area of viable
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31 myocardial islands within the infarcted tissue (0.09 ± 0.02 mm², $p=0.035$) was
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33 significantly larger than the viable area in hearts treated with the Control-MSCs group
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35 and VEGF group (0.03 ± 0.01 mm², 0.04 ± 0.02 mm², respectively) (Figure 8).
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45 **3.5 Echocardiographic assessment of the enhancements in cardiac function after**

46 **MI**

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50 Cardiac function was evaluated using echocardiography. For the LbL-MSCs
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52 group, at 3 days and weeks 1, 3, 6, the LVSD, LVDD, EDV, and ESV were obviously
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54 smaller, and the indexes of fractional shortening and ejection fraction were
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1 significantly restored compared to the Control-MSCs group and VEGF group,
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3 suggesting contractile improvement (Figure 9).
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6 An example of pulsed-wave Doppler recordings of mitral inflow is shown in
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8 LbL-MSCs group, Control-MSCs group and VEGF group. Ischemia/reperfusion
9 increased both early diastolic filling (E-wave) velocity and atrial filling (A-wave)
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11 velocity and therefore resulted in a significant decrease in the ratio of E-wave to
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13 A-wave velocity (E/A) relative to baseline values in the base animals. The results
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15 show that cardiac diastolic function were significantly restored compared to the
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17 Control-MSCs group and VEGF group (Figure 9).
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28 **Discussion**

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31 VEGF is the most important angiogenesis factor for enhancing cardiac function
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33 [4]. However, previous studies have shown that only a large dose of VEGF can
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35 modestly improve angiogenesis and cardiac function and can lead to serious side
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37 effects [37, 38]. Therefore, targeted VEGF delivery to the infarcted zone using an
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39 adequate therapeutic dose of VEGF has become a popular research topic.
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45 In this study, we first developed a new targeting MSCs carrier using a
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47 VEGF-loaded gelatin and alginate polyelectrolyte assembly. MSCs were encapsulated
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49 using a layer-by-layer self-assembly technique, which offers the interaction of
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51 opposite charges. The outcome of this technique was to have multiple layers of MSCs
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53 on the cell surface.
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1 The question was raised as to whether a single MSCs-based LbL encapsulation
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3 with gelatin and alginate would influence the tropism of MSCs. A previous report
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5 showed that LbL encapsulation with gelatin and alginate polyelectrolytes did not
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7 affect the viability, proliferation of these cells [27].
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11 The results reveal that LbL encapsulation does not significantly influence MSCs
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13 viability over a dynamic period. In summary, gelatin and alginate are suitable for LbL
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15 encapsulations of MSCs. VEGF-loaded LbL-MSCs have better survival compared to
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17 MSCs and Control-MSCs. VEGF is a potent angiogenic growth factor that promotes
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19 endothelial cells (ECs) survival. The anti-apoptotic signaling pathway associated with
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21 VEGF signaling is mediated through the phosphorylation of AKT and results in a
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23 reduction in caspase 3 cleavage. It is well known that the MSCs differentiation
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25 potential occurs according to three lineages, osteogenic, chondrogenic and adipogenic
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27 [39]. Osteogenic differentiation of mesenchymal stromal cells was shown for
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29 LbL-MSCs and Control-MSCs compared to MSCs. Previous studies have shown that
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31 osteocalcin is significantly increased under hypoxia or exogenous VEGF [40, 41].
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42 Favorable tropism of LbL-MSCs was demonstrated in vitro using a co-culture
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44 system. In vivo, the results showed that the MI tropism of the LbL-MSCs group was
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46 well preserved, despite poorer results for the MSCs group. The thin LbL film on the
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48 LbL-MSCs had no influence on migration. Thus, we believe that the tropism of
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50 LbL-MSCs was slightly weakened by the barrier of the SDF-1/CXCR4 interaction,
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52 which resulted from the thin LbL film. The isoelectric point (IEP) of gelatin (type A)
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54 is 7-9. Based on its positive charge under certain conditions of pH, gelatin is an
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1 interesting candidate for use in effective intracellular delivery. Previous studies have
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3 shown that DNA, protein and other intracellular molecules have also been delivered
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5 in this way [42, 43]. Gelatin could deliver VEGF to the cytoplasm. MSCs
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7 internalization by endocytosis promoted by the LbL films is important for enhancing
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9 the chemotaxis of MSCs. The internalization of partial LbL films could promote the
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12 the chemotaxis of MSCs. The internalization of partial LbL films could promote the
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14 time-dependent binding of chemokines and MSCs.
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17 The porous construction of the self-assembled layer may have played a vital
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19 role by reducing the barriers to SDF-1/CXCR4 interaction. Improvement in MI
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21 tropism was observed for the LbL-MSCs group with further culturing after 1 week.
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23 The self-assembled layer may be a barrier that influences SDF-1/CXCR4 interaction.
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25 With the decomposition of the self-assembled layers, the binding of SDF-1 and
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27 CXCR4 became easier, and the tropism of the LbL-MSCs increased significantly.
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29 This result also may be associated with MSCs endocytosis of the self-assembled
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31 layers, resulting in an easier binding of SDF-1 and CXCR4 on the LbL-MSCs [44].
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35 The LbL self-assembly technique has been studied for drug delivery in numerous
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37 studies [28, 29]. Insulin [30], doxorubicin [31], and bone morphogenetic protein-2
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39 (BMP-2) have been reported to be loaded onto the LbL structure, with the distinct
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41 effect of sustained release [45]. A large dose of VEGF (1-30 $\mu\text{g/kg}$ animal weight)
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43 has previously been required for systemic administration, leading to a modest
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45 improvement in vascular density in the MI region [37, 38], which may be due to its
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47 short half-life (approximately several minutes) in plasma [7]. This half-life of VEGF
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49 could be enhanced by LbL self-assembled thin films, where encapsulated VEGF is
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protected from degradation in the circulation. In our studies, a single dose of 10 ug VEGF-encapsulated MSCs resulted in significant improvements in cardiac function and vascular structure up to 6 weeks after MI. Moreover, we found that there was improvement in systolic and diastolic function at an early stage; however, angiogenesis did not occur. This finding may be related to the VEGF of myocardial apoptosis and cardiac remodeling [12, 46, 47] or other factors secreted by MSCs [33, 48, 49], although angiogenesis does not occur in the early stage. A significant increase in angiogenesis was found after 2 weeks, as has been reported previously [16, 50]. This increase is related to the expression of VEGF receptors on endothelial cells. In the early stage (3 days post-MI), the expression of receptors decreased to some extent. However, at days 7, VEGF receptor expression was active, and cells had moved from the distant non-infarcted zone to the border zone as a part of the repair process [51, 52].

Our experimental results indicated that VEGF release from LbL-MSCs occurred in a time-dependent and pH-dependent manner in vitro, and a pH value of 6.5 was used to mimic the ischemic environment. A low pH is an important characteristic of MI [53], and the acidic environment of the infarct area could prolong the release of VEGF to improve angiogenesis, which is in agreement with a previous report [27]. The reason for this improvement may be that a pH of 6.5 is closer to the middle of the isoelectric point (IEP) between alginate and gelatin than a pH of 7.4 [54, 55], making the LbL thin films more stable at a pH of 6.5 than at a pH of 7.4. Another explanation may be that the permeability of LbL thin films is changed at a different pH [56].

1 Gelatin and alginate polyelectrolytes were used in this research. Gelatin, which is
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3 degraded from collagen, is a biocompatible protein that is similar to the native
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5 extracellular matrix [57]. Alginate is widely used in tissue engineering and has shown
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7 good biocompatibility [58, 59]. As such, gelatin and alginate were ideal biomaterials
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9 for LbL encapsulation.
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14 In summary, VEGF-encapsulated MSCs provided a safer and more effective
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16 means of regenerating and maintaining vasculature and cardiac function by targeting
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18 VEGF delivery to diseased cardiac tissue, based on the tropism of MSCs and
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20 layer-by-layer self-assembly. Clearly, further studies are needed to optimize this novel
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22 delivery method to improve the tropism of LbL-MSCs.
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31 **Conclusions**

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34 We developed a VEGF delivery system targeting MI tissue to improve
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36 angiogenesis and cardiac function based on the tropism of MSCs by using
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38 layer-by-layer self-assembly. The results showed that VEGF-encapsulated MSCs had
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40 a sustained release of VEGF and tropism to SDF-1 in vitro. In vivo,
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42 VEGF-encapsulated MSCs exhibited homing to MI tissue, enhanced cardiac function,
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44 perfused the infarct area and promoted angiogenesis. These preclinical findings
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46 suggest that VEGF-loaded layer-by-layer self-assembled encapsulated MSCs may be
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48 a promising minimally invasive therapy for treating MI. Compared to other drug
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50 delivery systems, the novel drug delivery systems based on tropism of MSCs and
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52 layer-by-layer self-assembly have some advantages: 1) MSCs are inherent cells and
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1 have been used in cell therapy and drug delivery systems based on their favorable
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3 biocompatibility; 2) the drug was deposited on the cell surface using layer-by-layer
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5 (LbL) self-assembly and exhibited sustained release and good pharmaceutical
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7 activity; and 3) other drugs loaded to layer-by-layer self-assembled encapsulated
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9 MSCs may be promising therapies for treating other diseases.
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36 **Additional Information**

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39 The authors declare that they have no competing financial interests.
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References

- [1] P. American College of Emergency, A. Society for Cardiovascular, Interventions, P.T. O'Gara, F.G. Kushner, D.D. Ascheim, D.E. Casey, Jr., M.K. Chung, J.A. de Lemos, S.M. Ettinger, J.C. Fang, F.M. Fesmire, B.A. Franklin, C.B. Granger, H.M. Krumholz, J.A. Linderbaum, D.A. Morrow, L.K. Newby, J.P. Ornato, N. Ou, M.J. Radford, J.E. Tamis-Holland, C.L. Tommaso, C.M. Tracy, Y.J. Woo, D.X. Zhao, J.L. Anderson, A.K. Jacobs, J.L. Halperin, N.M. Albert, R.G. Brindis, M.A. Creager, D. DeMets, R.A. Guyton, J.S. Hochman, R.J. Kovacs, F.G. Kushner, E.M. Ohman, W.G. Stevenson, C.W. Yancy, 2013 ACCF/AHA guideline for the management of ST-elevation myocardial infarction: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines, *Journal of the American College of Cardiology* 61(4) (2013) e78-140.
- [2] E.T. Chouchani, V.R. Pell, E. Gaude, D. Aksentijevic, S.Y. Sundier, E.L. Robb, A. Logan, S.M. Nadtochiy, E.N. Ord, A.C. Smith, F. Eyassu, R. Shirley, C.H. Hu, A.J. Dare, A.M. James, S. Rogatti, R.C. Hartley, S. Eaton, A.S. Costa, P.S. Brookes, S.M. Davidson, M.R. Duchon, K. Saeb-Parsy, M.J. Shattock, A.J. Robinson, L.M. Work, C. Frezza, T. Krieg, M.P. Murphy, Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS, *Nature* 515(7527) (2014) 431-5.
- [3] I. Zachary, R.D. Morgan, Therapeutic angiogenesis for cardiovascular disease: biological context, challenges, prospects, *Heart* 97(3) (2011) 181-9.
- [4] T.D. Crafts, A.R. Jensen, E.C. Blocher-Smith, T.A. Markel, Vascular endothelial growth factor: therapeutic possibilities and challenges for the treatment of ischemia, *Cytokine* 71(2) (2015) 385-93.
- [5] C.H. Li, B. Liu, Z.H. Dai, Y.X. Tao, Inhibition of vascular endothelial growth factor receptor-1(VEGFR-1) function as a potential therapy for clear cell renal cell carcinoma (CCRCC), *Cancer Res* 69 (2009).
- [6] C. de Vries, J.A. Escobedo, H. Ueno, K. Houck, N. Ferrara, L.T. Williams, The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor, *Science* 255(5047) (1992) 989-91.
- [7] J.S. Rudge, J. Holash, D. Hylton, M. Russell, S. Jiang, R. Leidich, N. Papadopoulos, E.A. Pyles, A. Torri, S.J. Wiegand, G. Thurston, N. Stahl, G.D. Yancopoulos, VEGF Trap complex formation measures production rates of VEGF, providing a biomarker for predicting efficacious angiogenic blockade, *Proceedings of the National Academy of Sciences of the United States of America* 104(47) (2007) 18363-70.
- [8] S.E. Epstein, R. Kornowski, S. Fuchs, H.F. Dvorak, Angiogenesis therapy: amidst the hype, the neglected potential for serious side effects, *Circulation* 104(1) (2001) 115-9.
- [9] M. Inoue, H. Itoh, M. Ueda, T. Naruko, A. Kojima, R. Komatsu, K. Doi, Y. Ogawa, N. Tamura, K. Takaya, T. Igaki, J. Yamashita, T.H. Chun, K. Masatsugu, A.E. Becker, K. Nakao, Vascular endothelial growth factor (VEGF) expression in human coronary atherosclerotic lesions: possible pathophysiological significance of VEGF in progression of atherosclerosis, *Circulation* 98(20) (1998) 2108-16.

- [10] R.J. Lee, M.L. Springer, W.E. Blanco-Bose, R. Shaw, P.C. Ursell, H.M. Blau, VEGF gene delivery to myocardium: deleterious effects of unregulated expression, *Circulation* 102(8) (2000) 898-901.
- [11] A. Pettersson, J.A. Nagy, L.F. Brown, C. Sundberg, E. Morgan, S. Jungles, R. Carter, J.E. Krieger, E.J. Manseau, V.S. Harvey, I.A. Eckelhoefer, D. Feng, A.M. Dvorak, R.C. Mulligan, H.F. Dvorak, Heterogeneity of the angiogenic response induced in different normal adult tissues by vascular permeability factor/vascular endothelial growth factor, *Laboratory investigation; a journal of technical methods and pathology* 80(1) (2000) 99-115.
- [12] J.M. Rosano, R. Cheheltani, B. Wang, H. Vora, M.F. Kiani, D.L. Crabbe, Targeted Delivery of VEGF after a Myocardial Infarction Reduces Collagen Deposition and Improves Cardiac Function, *Cardiovascular engineering and technology* 3(2) (2012) 237-247.
- [13] R.C. Scott, D. Crabbe, B. Krynska, R. Ansari, M.F. Kiani, Aiming for the heart: targeted delivery of drugs to diseased cardiac tissue, *Expert opinion on drug delivery* 5(4) (2008) 459-70.
- [14] F. Yang, P. Chen, W. He, N. Gu, X. Zhang, K. Fang, Y. Zhang, J. Sun, J. Tong, Bubble microreactors triggered by an alternating magnetic field as diagnostic and therapeutic delivery devices, *Small* 6(12) (2010) 1300-5.
- [15] Y. Tang, X. Gan, R. Cheheltani, E. Curran, G. Lamberti, B. Krynska, M.F. Kiani, B. Wang, Targeted delivery of vascular endothelial growth factor improves stem cell therapy in a rat myocardial infarction model, *Nanomedicine* 10(8) (2014) 1711-8.
- [16] R.C. Scott, J.M. Rosano, Z. Ivanov, B. Wang, P.L. Chong, A.C. Issekutz, D.L. Crabbe, M.F. Kiani, Targeting VEGF-encapsulated immunoliposomes to MI heart improves vascularity and cardiac function, *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 23(10) (2009) 3361-7.
- [17] J. Zhang, L. Ding, Y. Zhao, W. Sun, B. Chen, H. Lin, X. Wang, L. Zhang, B. Xu, J. Dai, Collagen-targeting vascular endothelial growth factor improves cardiac performance after myocardial infarction, *Circulation* 119(13) (2009) 1776-84.
- [18] T.T. Lau, D.A. Wang, Stromal cell-derived factor-1 (SDF-1): homing factor for engineered regenerative medicine, *Expert opinion on biological therapy* 11(2) (2011) 189-97.
- [19] B.R. Son, L.A. Marquez-Curtis, M. Kucia, M. Wysoczynski, A.R. Turner, J. Ratajczak, M.Z. Ratajczak, A. Janowska-Wieczorek, Migration of bone marrow and cord blood mesenchymal stem cells in vitro is regulated by stromal-derived factor-1-CXCR4 and hepatocyte growth factor-c-met axes and involves matrix metalloproteinases, *Stem Cells* 24(5) (2006) 1254-1264.
- [20] C. Steingen, F. Brenig, L. Baumgartner, J. Schmidt, A. Schmidt, W. Bloch, Characterization of key mechanisms in transmigration and invasion of mesenchymal stem cells, *Journal of molecular and cellular cardiology* 44(6) (2008) 1072-84.
- [21] V.Y. Leung, D.M. Aladin, F. Lv, V. Tam, Y. Sun, R.Y. Lau, S.C. Hung, A.H. Ngan, B. Tang, C.T. Lim, E.X. Wu, K.D. Luk, W.W. Lu, K. Masuda, D. Chan, K.M. Cheung, Mesenchymal stem cells reduce intervertebral disc fibrosis and facilitate repair, *Stem Cells* 32(8) (2014) 2164-77.

- [22] D. Li, N. Wang, L. Zhang, Z. Hanyu, B. Xueyuan, B. Fu, C. Shaoyuan, W. Zhang, S. Xuefeng, R. Li, X. Chen, Mesenchymal stem cells protect podocytes from apoptosis induced by high glucose via secretion of epithelial growth factor, *Stem cell research & therapy* 4(5) (2013) 103.
- [23] C.H. Ryu, S.A. Park, S.M. Kim, J.Y. Lim, C.H. Jeong, J.A. Jun, J.H. Oh, S.H. Park, W.I. Oh, S.S. Jeun, Migration of human umbilical cord blood mesenchymal stem cells mediated by stromal cell-derived factor-1/CXCR4 axis via Akt, ERK, and p38 signal transduction pathways, *Biochemical and biophysical research communications* 398(1) (2010) 105-10.
- [24] J.L. Carter, I. Drachuk, S. Harbaugh, N. Kelley-Loughnane, M. Stone, V.V. Tsukruk, Truly nonionic polymer shells for the encapsulation of living cells, *Macromolecular bioscience* 11(9) (2011) 1244-53.
- [25] A.L. Hillberg, M. Tabrizian, Biorecognition through layer-by-layer polyelectrolyte assembly: In-situ hybridization on living cells, *Biomacromolecules* 7(10) (2006) 2742-2750.
- [26] S.H. Yang, S.M. Kang, K.B. Lee, T.D. Chung, H. Lee, I.S. Choi, Mussel-inspired encapsulation and functionalization of individual yeast cells, *Journal of the American Chemical Society* 133(9) (2011) 2795-7.
- [27] W. Li, T. Guan, X. Zhang, Z. Wang, M. Wang, W. Zhong, H. Feng, M. Xing, J. Kong, The Effect of Layer-by-Layer Assembly Coating on the Proliferation and Differentiation of Neural Stem Cells, *ACS applied materials & interfaces* 7(5) (2015) 3018-29.
- [28] U. Manna, S. Patil, Dual drug delivery microcapsules via layer-by-layer self-assembly, *Langmuir : the ACS journal of surfaces and colloids* 25(18) (2009) 10515-22.
- [29] A.N. Zelikin, Drug releasing polymer thin films: new era of surface-mediated drug delivery, *Acs Nano* 4(5) (2010) 2494-509.
- [30] K. Yoshida, R. Hashide, T. Ishii, S. Takahashi, K. Sato, J. Anzai, Layer-by-layer films composed of poly(allylamine) and insulin for pH-triggered release of insulin, *Colloids and surfaces. B, Biointerfaces* 91 (2012) 274-9.
- [31] H.J. Shen, H. Shi, M. Xie, K. Ma, B. Li, S. Shen, X.S. Wang, Y. Jin, Biodegradable chitosan/alginate BSA-gel-capsules for pH-controlled loading and release of doxorubicin and treatment of pulmonary melanoma, *J Mater Chem B* 1(32) (2013) 3906-3917.
- [32] T.G. Shutava, S.S. Balkundi, P. Vangala, J.J. Steffan, R.L. Bigelow, J.A. Cardelli, D.P. O'Neal, Y.M. Lvov, Layer-by-Layer-Coated Gelatin Nanoparticles as a Vehicle for Delivery of Natural Polyphenols, *Acs Nano* 3(7) (2009) 1877-1885.
- [33] M. Cai, R. Shen, L. Song, M. Lu, J. Wang, S. Zhao, Y. Tang, X. Meng, Z. Li, Z.X. He, Bone Marrow Mesenchymal Stem Cells (BM-MSCs) Improve Heart Function in Swine Myocardial Infarction Model through Paracrine Effects, *Scientific reports* 6 (2016) 28250.
- [34] P. Chomczynski, A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples, *Biotechniques* 15(3) (1993) 532-4, 536-7.

- [35] R. Gaertner, M.P. Jacob, F. Prunier, E. Angles-Cano, J.J. Mercadier, J.B. Michel, The plasminogen-MMP system is more activated in the scar than in viable myocardium 3 months post-MI in the rat, *Journal of molecular and cellular cardiology* 38(1) (2005) 193-204.
- [36] M. Yin, I.C. van der Horst, J.P. van Melle, C. Qian, W.H. van Gilst, H.H. Sillje, R.A. de Boer, Metformin improves cardiac function in a nondiabetic rat model of post-MI heart failure, *American journal of physiology. Heart and circulatory physiology* 301(2) (2011) H459-68.
- [37] J.J. Lopez, R.J. Laham, A. Stamler, J.D. Pearlman, S. Bunting, A. Kaplan, J.P. Carrozza, F.W. Sellke, M. Simons, VEGF administration in chronic myocardial ischemia in pigs, *Cardiovascular research* 40(2) (1998) 272-81.
- [38] S.B. Freedman, J.M. Isner, Therapeutic angiogenesis for ischemic cardiovascular disease, *Journal of molecular and cellular cardiology* 33(3) (2001) 379-93.
- [39] C. Yang, H. Frei, F.M. Rossi, H.M. Burt, The differential in vitro and in vivo responses of bone marrow stromal cells on novel porous gelatin-alginate scaffolds, *J Tissue Eng Regen Med* 3(8) (2009) 601-14.
- [40] Y. Wang, J. Li, Y. Wang, L. Lei, C. Jiang, S. An, Y. Zhan, Q. Cheng, Z. Zhao, J. Wang, L. Jiang, Effects of hypoxia on osteogenic differentiation of rat bone marrow mesenchymal stem cells, *Mol Cell Biochem* 362(1-2) (2012) 25-33.
- [41] K. Joensuu, L. Uusitalo, J.J. Alm, H.T. Aro, T.A. Hentunen, T.J. Heino, Enhanced osteoblastic differentiation and bone formation in co-culture of human bone marrow mesenchymal stromal cells and peripheral blood mononuclear cells with exogenous VEGF, *Orthop Traumatol Surg Res* 101(3) (2015) 381-6.
- [42] T. Guo, J. Zhao, J. Chang, Z. Ding, H. Hong, J. Chen, J. Zhang, Porous chitosan-gelatin scaffold containing plasmid DNA encoding transforming growth factor-beta1 for chondrocytes proliferation, *Biomaterials* 27(7) (2006) 1095-103.
- [43] K. Wang, X. Chen, Y. Pan, Y. Cui, X. Zhou, D. Kong, Q. Zhao, Enhanced vascularization in hybrid PCL/gelatin fibrous scaffolds with sustained release of VEGF, *Biomed Res Int* 2015 (2015) 865076.
- [44] E.B. Eaton, Jr., T.R. Varney, Mesenchymal stem cell therapy for acute radiation syndrome: innovative medical approaches in military medicine, *Military Medical Research* 2 (2015) 2.
- [45] T. Crouzier, K. Ren, C. Nicolas, C. Roy, C. Picart, Layer-By-Layer Films as a Biomimetic Reservoir for rhBMP-2 Delivery: Controlled Differentiation of Myoblasts to Osteoblasts, *Small* 5(5) (2009) 598-608.
- [46] Y. Devaux, M. Vausort, F. Azuaje, M. Vaillant, M.L. Lair, E. Gayat, J. Lassus, L.L. Ng, D. Kelly, D.R. Wagner, I.B. Squire, Low levels of vascular endothelial growth factor B predict left ventricular remodeling after acute myocardial infarction, *Journal of cardiac failure* 18(4) (2012) 330-7.
- [47] K.Y. Howangyin, I. Zlatanova, C. Pinto, A. Ngkelo, C. Cochain, M. Rouanet, J. Vilar, M. Lemitre, C. Stockmann, B.K. Fleischmann, Z. Mallat, J.S. Silvestre, Myeloid-Epithelial-Reproductive Receptor Tyrosine Kinase and Milk Fat Globule Epidermal Growth Factor 8 Coordinately Improve Remodeling After Myocardial

Infarction via Local Delivery of Vascular Endothelial Growth Factor, *Circulation* 133(9) (2016) 826-39.

[48] A.A. Mangi, N. Noiseux, D. Kong, H. He, M. Rezvani, J.S. Ingwall, V.J. Dzau, Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts, *Nature medicine* 9(9) (2003) 1195-201.

[49] A. Saxena, J.E. Fish, M.D. White, S. Yu, J.W.P. Smyth, R.M. Shaw, J.M. DiMaio, D. Srivastava, Stromal cell-derived factor-1 alpha is cardioprotective after myocardial infarction, *Circulation* 117(17) (2008) 2224-2231.

[50] K. Albrecht-Schgoer, W. Schgoer, J. Holfeld, M. Theurl, D. Wiedemann, C. Steger, R. Gupta, S. Semsroth, R. Fischer-Colbrie, A.G. Beer, U. Stanzl, E. Huber, S. Misener, D. Dejaco, R. Kishore, O. Pachinger, M. Grimm, N. Bonaros, R. Kirchmair, The angiogenic factor secretoneurin induces coronary angiogenesis in a model of myocardial infarction by stimulation of vascular endothelial growth factor signaling in endothelial cells, *Circulation* 126(21) (2012) 2491-501.

[51] X.H. Wang, G.P. Zhang, H.M. Jin, S.F. Chen, Dynamic changes in the expression of growth factor receptors in the myocardium microvascular endothelium after murine myocardial infarction, *Chinese medical journal* 120(6) (2007) 485-90.

[52] A. Suhardja, H. Hoffman, Role of growth factors and their receptors in proliferation of microvascular endothelial cells, *Microsc Res Techniq* 60(1) (2003) 70-75.

[53] M. von Ardenne, P.G. Reitnauer, K. Rohde, [Changes in myocardial pH and their significance for the treatment of myocardial infarction and multi-stage cancer therapy], *Wiener klinische Wochenschrift* 84(3) (1972) 47-54.

[54] D.S. Jackson, A. Neuberger, Observations on the isoionic and isoelectric point of acid-processed gelatin from insoluble and citrate-extracted collagen, *Biochimica et biophysica acta* 26(3) (1957) 638-9.

[55] M. Cegnar, J. Kerc, Self-assembled Polyelectrolyte Nanocomplexes of Alginate, Chitosan and Ovalbumin, *Acta chimica Slovenica* 57(2) (2010) 431-41.

[56] K. Sato, K. Yoshida, S. Takahashi, J.I. Anzai, pH- and sugar-sensitive layer-by-layer films and microcapsules for drug delivery, *Adv Drug Deliver Rev* 63(9) (2011) 809-821.

[57] H.A. Wang, M.B. Hansen, D.W.P.M. Lowik, J.C.M. van Hest, Y.B. Li, J.A. Jansen, S.C.G. Leeuwenburgh, Oppositely Charged Gelatin Nanospheres as Building Blocks for Injectable and Biodegradable Gels, *Adv Mater* 23(12) (2011) H119-H124.

[58] C. Sharma, A.K. Dinda, P.D. Potdar, C.F. Chou, N.C. Mishra, Fabrication and characterization of novel nano-biocomposite scaffold of chitosan-gelatin-alginate-hydroxyapatite for bone tissue engineering, *Materials science & engineering. C, Materials for biological applications* 64 (2016) 416-27.

[59] H. Baniasadi, S. Mashayekhan, S. Fadaoddini, Y. Haghirsharifzamani, Design, fabrication and characterization of oxidized alginate-gelatin hydrogels for muscle tissue engineering applications, *Journal of biomaterials applications* 31(1) (2016) 152-61.

Figure1

a

LbL-MSCs

b

MSCs

Figure2

Figure3

Figure4

Figure5

a

MSCs

Control-MSCs

LbL-MSCs (2d)

LbL-MSCs (7d)

b

Figure6

Figure7

Figure8

Figure9

Figure titles and legends

Figure 1. The characterization of LbL encapsulation using TEM. Different magnifications of TEM images of (a) LbL-MSCs and (b) untreated MSCs are shown. Arrows indicate the LbL thin film on the surface of LbL-MSCs.

Figure 2. The presence of VEGF on thin LbL film. (a, b, c) LbL-MSCs were stained with anti-VEGF (green) to illustrate the presence of VEGF on the cell surface on day 0, 2, 7. (d-i) Control-MSCs and MSCs were stained with anti-VEGF (green) and DAPI to illustrate the no presence of VEGF on the cell surface. (j) The zeta-potential of nanocoated MSCs is shown. The groups include untreated MSCs, MSCs nanocoated with gelatin, MSCs nanocoated with gelatin/alginate, and MSCs nanocoated with (gelatin)₂/alginate. Scale bar: 20 μ M.

Figure 3. Morphological changes of MSCs and LbL-MSCs. (a) Morphological changes of LbL-MSCs and MSCs detected using SEM are shown on days 1, 3, 5. (b) F-actin staining by phalloidin showed the morphological changes on the cytoskeleton of LbL-MSCs compared to MSCs. The cytoskeleton of LbL-MSCs was much more restrained than that of MSCs. (c) The spreading area of each cell was quantified using ImageJ based on the SEM results; * $p < 0.01$. Scale bar: 20 μ M.

Figure 4. The presence of SDF-1/CXCR-4 on LbL-MSCs. In vitro tropism of LbL-MSCs was slightly weakened in Transwell experiments. (a) The presence of

SDF-1/CXCR-4 on LbL-MSCs and MSCs is shown. LbL-MSCs were stained with SDF-1 (red) to illustrate the presence of SDF-1/CXCR4 (red) on the cell surface. Scale bar: 10 μ m. (b) Scratch assays showed that no significant difference was observed for the migration of MSCs. (c) The co-culture system of Transwell 24-well experiments showed that the tropism of LbL-MSCs to SDF-1 was slightly weakened on days 1, 3 and 7 compared to MSCs. (d) The tropism of the MSCs was approximately 3 times stronger than LbL-MSCs at 1 d, 1.5 times stronger than LbL-MSCs at 3 days, and not significant at 7 days and 10 days. Scale bar: 20 μ M.

Figure 5. Recruitment of LbL-MSCs into the infarct zone was slightly weakened depending on culture time in vivo. (a) Positive MSCs (labeled red) are shown in the VEGF group, LbL-MSCs group, and Control-MSCs group in the ischemic myocardium (n=3). (b) The change in the recruitment of LbL-MSCs, MSCs and Control-MSCs at different time points is shown. Scale bar: 20 μ M.

Figure 6. The cumulative VEGF release curve and VEGF deposits in the infarct area. (a) VEGF deposits in the infarct area in the VEGF group, LbL-MSCs group, and Control-MSCs group by western blot are shown. (b) MSCs and LbL-MSCs were cultured under different pH values in 24-well plates (n=3). The cumulative release curve of VEGF was obtained using an ELISA kit; *p<0.05.

Figure 7. The perfused infarct area is shown using in vivo micro-CT. The border zone of the myocardium in MI rats at 4 weeks post-MI was analyzed using micro-CT. The groups were as follows: (a) untreated mice (n=9), (b) LbL-MSCs group (n=9), (c) Control-MSCs group (n=9), and (d) VEGF group (n=9). (e) The border zone of the myocardium in the different groups is shown.

Figure 8. Angiogenesis induced by LbL-MSCs. (a, b, c) The exact vascular densities in the ischemic myocardium for the VEGF group, Control-MSCs group and LbL-MSCs group are shown on weeks 4. (d, e, f) An increased average area of cardiomyocyte islands is shown in the ischemic myocardium of the VEGF group, Control-MSCs group and LbL-MSCs group on weeks 4 (*p <0.05). Scale bar: 20 μ M.

Figure 9. Effects on cardiac function. (a, b, c, d) Cardiac systolic function was detected by echocardiography using a 2-dimensional guided M-mode ultrasound system in the Control-MSCs group (solid triangles), Base group, VEGF group (solid squares) and the LbL-MSCs group (open circles). (e, f, g, h) Cardiac diastolic function was measured by a ratio of E-wave to A-wave velocity (E/A ratio [normal, $1 < \text{E/A ratio} < 2$]) in the Control-MSCs group (solid triangles), Base group, VEGF group (solid squares) and the LbL-MSCs group (open circles).