Functional Nanocomplexes with Vascular Endothelial Growth Factor A/C Isoforms Improve Collateral Circulation and Cardiac Function

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1. Introduction

Caused by acute occlusion of coronary arteries, myocardial infarction (MI) mainly leads to cardiac dysfunction or even death.[1] Clinically, percutaneous coronary intervention (PCI) is performed to treat MI by restoring blood supply to the ischemic area.[2] Studies have shown that new microvessels can compensate blood and oxygen supplies and restore the injured myocardium.[3] Notably, the incidence of heart failure and death after PCI treatment is reduced significantly in patients with better collateral circulation.[4] Therefore, it has attracted considerable efforts to improve collateral circulation for patients undergone PCI.

The vascular endothelial growth factor (VEGF) family plays a central role in both angiogenesis and vasculogenesis.[5] VEGF-A is capable of promoting angiogenesis by binding to the VEGFR-1 receptor and regulating the proliferation of endothelial cells. However, effective delivery systems are needed because of their instability, immunogenicity, and so on. Crosslinked negatively charged heparin polysaccharide nanoparticle (HepNP) is proposed for protein delivery. HepNP can efficiently condense vascular endothelial growth factor (VEGF) because of the unique electronegative sulfonic acid and carboxyl domain of heparin. HepNP is then assembled with VEGF-C (Hep@VEGF-C) or VEGF-A (Hep@VEGF-A) protein for the therapy of myocardial infarction (MI) via intravenous injection. Hep@VEGF-A-mediated improvement of cardiac function by promoting angiogenesis is limited because of elevated vascular permeability, while Hep@VEGF-C effectively promotes lymphangiogenesis and reduces edema. On this basis, a graded delivery of VEGF-C (0.5–1 h post-MI) and VEGF-A (5 d post-MI) using HepNP is developed. At the dose ratio of 3:1 (Hep@VEGF-C vs Hep@VEGF-A), Hep@VEGF functional complexes substantially reduce the scar formation (≈−39%; p < 0.05) and improve cardiac function (≈+74%; p < 0.05). Such a HepNP delivery system provides a simple and effective therapeutic strategy for cardiovascular diseases by delivering functional proteins. Because of the unique binding ability of heparin with cytokines and growth factors, HepNP also has considerable application prospects in protein therapy for other serious diseases.
VEGF-A has been used for treating ischemic diseases, such as coronary, myocardial, cerebral, and bowel ischemia. Although these efforts have achieved certain therapeutic effects in animal studies, there are limitations when migrating these results to human subjects. A number of clinical trials have shown that VEGF-A caused elevated permeability, tissue edema, etc. Reducing edema is a critical criterion for VEGF-A based therapy.

Lymphatics are blind-ended, one-way absorption vessels that transport interstitial fluid/immune cells to lymph nodes and deliver them back to the venous system and can be introduced to reduce edema. Lymphatic obstruction leads to the development of edema and aggravates cardiac dysfunction. Reperfusion after PCI further aggravates myocardial injury and cardiac edema. VEGF-C stimulates lymphangiogenesis by acting on VEGFR-3 receptor sites. Earlier studies have proved that VEGF-C protein encapsulated by microparticles and injected into the heart can significantly promote the formation of lymphatic vessels and reduce heart injury.

Delivering pharmacologically active proteins to specific tissues or cells engenders various challenges, such as instability and immunogenicity. Thus, the development of safe and effective protein delivery systems is needed urgently. Negatively charged heparin, a well-known clinical anticoagulant and thrombolytic drug, can interact with many functional proteins, including most chemokines and growth factors, through its unique electronegative sulfonic acid and carboxyl domain. These interactions can target the peptides to specific sites in tissues, improving their stability and function.

In this study, HepNPs were assembled with VEGF-C protein (lymphangiogenesis factor) into Hep@VEGF-C complexes and with VEGF-A protein (angiogenesis factor) into Hep@VEGF-A complexes for an intravenous (iv) MI therapy. Hep@VEGF-C was administered at the acute phase (within 0.5–1 h post-MI) to promote lymphangiogenesis, which efficiently eliminated the edema and vascular leakage. Hep@VEGF-A was administered 5 d post-MI to promote angiogenesis, which restored the blood supply. The staged delivery at a specific dose ratio of VEGF-C and VEGF-A proteins (Hep@VEGF-C:Hep@VEGF-A, 3:1) was used to achieve better therapeutic results. The HepNP-based protein delivery system administered via iv injection provides improved therapy performances while greatly reducing the requisite protein doses. The present study thus provides a powerful and economical strategy for the treatment of cardiovascular diseases.

2. Results and Discussion

2.1. Construction of Functional HepNP Complexes with VEGF-C Protein

HepNP was structurally designed and synthesized to be assembled around either VEGF-A or VEGF-C proteins as an effective protein therapy for MI. Heparin polysaccharide was used to prepare HepNPs via a redox reaction of lipoic acid, as previously reported. The preparation of HepNP was characterized, shown in Figure 1a,b.

We selected the VEGF-C protein as a representative member of the VEGF protein family. First, HepNP was assembled with VEGF-C at various weight ratios (W_{HepNP}:W_{VEGF-C} of 50:6 or 100:6, denoted as Hep(1/2)@VEGF-C and Hep@VEGF-C, respectively). Heparin polysaccharide was also mixed with the VEGF-C protein at a weight ratio of 100:6 (Heparin@VEGF-C). Particle sizes and zeta potentials of these complexes were characterized. Hep@VEGF-C complexes displayed a much smaller particle size (~150 nm) compared with HepNP (~230 nm), Hep(1/2)@VEGF-C (~250 nm), and Heparin@VEGF-C (~230 nm) (Figure 1a). Atomic force microscope (AFM) images confirmed these results (Figure 1b). Moreover, the AFM results implied that the HepNP-based complexes were more stable than complexes prepared with heparin. Hep@VEGF-C (~3 mV) and Hep(1/2)@VEGF-C (~6 mV) complexes had more positively charged surface potentials than did Heparin@VEGF-C (~12 mV) and HepNP (~23 mV) (Figure 1a); this surface charge may promote the binding of the complexes to the negatively charged cell membrane. However, the positive charge of Hep(1/2)@VEGF-C may also increase the risk of endocytosis, negatively impacting its binding to VEGFR-3 in cell membrane and decrease the therapy efficiency. Furthermore, Hep@VEGF-C complexes displayed a more uniform surface potential than Hep(1/2)@VEGF-C. These results suggested that the Hep@VEGF-C system may be more suitable for MI therapy. To further confirm the ability of the HepNP carrier to deliver VEGF-A proteins, Hep@VEGF-C complexes with a weight ratio of 100:6 (W_{HepNP}:W_{VEGF-C}) were also prepared and characterized (Figure 1a,b). These results proved that Hep@VEGF-C complexes (~155 nm, zeta ~5 mV) showed similar properties to Hep@VEGF-C (~150 nm, zeta ~3 mV).

To further investigate the binding to various delivery systems, VEGF-C was labeled with Cy7-SE prior to assembly with HepNP or heparin (Figure 1c; and Figure S1, Supporting Information). Hep@VEGF-C and Hep(1/2)@VEGF-C complexes displayed a higher binding affinity with human umbilical vein endothelial cells (HUVECs) than other complexes. Heparin binds to both VEGF and VEGFR, explaining the higher binding affinity of VEGF-C with heparin or HepNP. Moreover, the absolute values of the surface potentials for the HepNP-based complexes were small, which would benefit their approach to cells compared with more negatively charged Heparin@VEGF-C. Additionally, a greater amount of Hep(1/2)@VEGF-C complexes entered the cells than Hep@VEGF-C. Therefore, a greater protein enrichment on the cell surface was achieved by Hep@VEGF-C complexes than Hep(1/2)@VEGF-C; this was consistent with the surface potential results. Thus, HepNP was found to be more suitable for complexes with VEGF-C proteins, and the assembly at a weight ratio of 100:6 (Hep@VEGF-C) was more effective.

The release of the VEGF protein from its HepNP carrier was also observed under confocal laser scanning microscopy (CLSM) (Figure S2, Supporting Information). At the 4th h, the fluorescence localization showed that the protein (Red, labeled with Cy7-SE) bound to VEGFR together with HepNP (Green, labeled with fluorescein isothiocyanate (FITC)). The colocalization was pointed by the yellow arrow. Green fluorescence was hardly observed at the same location with the red fluorescence (pointed by the red arrow) at the 8th h, thus indicating the separation of HepNP carrier from the VEGF protein. Therefore, the VEGF protein binds to VEGFR in a cell membrane together.
with its HepNP carrier at the initial stage. As time progresses, VEGF proteins kept binding with VEGFR while the HepNP carrier shed from the delivery system, thereby guaranteeing the efficiencies of associated VEGF proteins.

2.2. Delivery of VEGF-C Protein with HepNP In Vivo

Similar to organ transplantation, an in vivo increase of foreign proteins may cause immune rejection. To confirm the safety
of HepNP-based complexes in vivo, VEGF-C protein, with and without HepNP vector, were injected into mice. Blood was collected 3 d after injection and CD3+/CD4+/CD8+ T lymphocytes were analyzed. With reference to the VEGF-C group, mice treated with Hep@VEGF-C functional complexes displayed lower CD3+ and CD8+ T lymphocyte percentages (Figure 2). Hep@VEGF-C complexes contained abundant heparin that may shield the VEGF-C protein from exposure to physiological environment, thus weakening the likelihood of immune rejection. These results indicated that the Hep@VEGF-C functional complexes did not increase the risk of immune rejection after injection and may even protect the protein from instigating immune rejection.

Next, in vivo animal experiments were performed to evaluate the efficacy of HepNP to transport proteins to target organs. First, the VEGF-C protein was labeled with fluorescent Cy7-SE for imaging. Myocardial cells immediately suffer from damage

Figure 1. a) Particle sizes and zeta potentials of HepNP nanocomplexes with or without VEGF protein (Hep(1/2)@VEGF-C, W_{HepNP}:W_{VEGF-C} = 50:6; Hep@VEGF-C, W_{HepNP}:W_{VEGF-C} = 100:6; Heparin@VEGF-C, W_{Heparin}:W_{VEGF-C} = 100:6, W_{HepNP}:W_{VEGF-A} = 100:6; n = 4 per group; Data: Mean ± SD). b) AFM images of HepNP with or without VEGF protein. c) Confocal fluorescence images show the binding situation of VEGF-C with HUVECs (VEGF-C protein was labeled with Cy7-SE).
due to ischemia and hypoxia after MI, and lymphangiogenesis occurs early and persistent 1–21 d after MI.[18] Patients with MI should be treated as soon as possible and simultaneously administration of drugs seems to promote recovery.[19] Therefore, actions that promote the reconstruction of lymphatic vessels to reduce the edema after MI are best taken as soon as possible. Thus, VEGF-C or Hep@VEGF-C functional complexes were iv injected into mice within 0.5–1 h post-MI.

Figure 2. Flow cytometry results of circulating T lymphocytes in mice 3 d after the injection of VEGF-C or Hep@VEGF-C complexes.
Vascular permeability in the heart after MI is typically elevated, which is beneficial for the accumulation of both VEGF-C and Hep@VEGF-C in the diseased area (Figure 3). Hep@VEGF-C showed significant accumulation in the heart after 1 h and was sustained much longer than the directly administered VEGF-C (Figure 3b). To confirm the results, heart tissues harvested at 72 h after injection were sectioned and observed by CLSM (Figure S3, Supporting Information).

Hep@VEGF-C showed a more obvious fluorescence signal than VEGF-C, further indicating the high efficiency of the HepNP-based delivery system. Compared with the physiologically electropositive VEGF-C protein, Hep@VEGF-C showed an electronegativity which inhibited the clearance of the loaded VEGF-C protein and increased its retention time in vivo. The limited efficiency of VEGF protein therapy is mainly due to its short residence time in the target organ; thus, a VEGF protein...
carried by vectors injected in situ after MI will increase efficiency, yet this procedure is likely operationally difficult.[6,11d] This HepNP-based delivery system can be administered via iv injection that provides effective accumulation in heart. Distributions in other organs were also detected (Figure S4, Supporting Information). The results showed that VEGF-C protein could efficiently be loaded with HepNP and, after administration, accumulated within to heart tissue.

2.3. MI Therapy Performance of Hep@VEGF-C Complexes

Ischemia resulting from MI leads to vascular permeability and edema, further resulting in cardiac damage.[11c,d,12] The promotion of early lymphangiogenesis helps to reduce edema and ultimately reduce post-MI cardiac damage. Among growth factors, VEGF-C promotes lymphangiogenesis by recognizing its receptor on the lymphatic endothelium.[11d] Thus, we selected the Hep@VEGF-C system for MI therapy during the acute phase.

Mice were treated with either VEGF-C protein or Hep@VEGF-C functional complexes (0.6 µg of VEGF-C protein) by iv injection within 0.5–1 h post-MI. Heart tissues were collected at 1 and 3 d post-MI for characterizations. First, lymphangiogenesis was characterized by immunofluorescence of lymphatic vessel-specific markers (VEGFR-3 and Prox-1).[11d] The labels in Figure 4 and Figure S5a (Supporting Information) represent the positive areas (VEGFR-3/red stain and Prox-1/green stain). Lymphatic vessels in the group that were treated with Hep@VEGF-C showed a significant increase when compared to the VEGF-C treatment group (Figure 4). No significant increase of lymphatic vessels in the HepNP group was observed compared to the control group (Figure 4 and Figure S5a, Supporting Information). These data imply that the HepNP-based complexes were capable of effectively and efficiently delivering VEGF-C protein to heart. These results may be attributable to the higher binding affinity and longer retention of VEGF-C proteins within the functional complexes (Figures 1c and 3; and Figures S1 and S2, Supporting Information). To examine whether the HepNP-based protein therapy could effectively reduce the edema caused by MI through promoting lymphangiogenesis,[11c,d,12] the myocardial water content was evaluated by the wet weight versus dry weight method (Figure S6, Supporting Information). The water content was significantly reduced by treatment with the Hep@VEGF-C complexes when compared with the control group (p < 0.05). This result is indicative of the Hep@VEGF-C complexes to significantly promote lymphangiogenesis and reduce the cardiac edema after MI.

Next, we examined the changes in cardiac function (Figures 5 and 6). When compared with the control group, the Hep@VEGF-C treatment group showed an improved restoration of heart functions through increases in the left ventricular ejection fraction (LVEF; p < 0.001) and left ventricular fractional shortening (LVFS; p < 0.001); the experimental group also showed decreases in the diastolic left ventricular internal diameter (LVIDD; p < 0.01) and systolic left ventricular internal dimension (LVIDS; p < 0.001) (Figure 5c). The heart weight (HW), body weight (BW), and tibia length (TL) of the mice were also measured to determine the heart-to-body weight ratio (HW/BW; p < 0.01) and heart weight-to-tibia length ratio (HW/TL; p < 0.001).[21] Compared with the control group, the HW/BW and HW/TL values were significantly reduced in the Hep@VEGF-C group (Figure 5b), indicating that compensatory cardiac remodeling was inhibited via Hep@VEGF-C based therapy. Fibrosis and pathological remodeling after MI lead to cardiac dysfunction or even heart failure. Thus, Red Sirius and Mason staining were used to measure cardiac fibrosis by calculating the ratio of the fibrotic scar area at the left ventricular wall area (Figure 6a,d,b,e).[22] In comparison with the control group, the Hep@VEGF-C group had smaller lesion areas. The compensatory hypertrophy of myocardial cells in the interface and distal areas of the MI was observed by wheat germ agglutinin (WGA) staining (Figure 6c,f). Hep@VEGF-C complex therapy reduced compensatory hypertrophy compared with the control. These results demonstrated that the HepNP vector can effectively deliver VEGF-C protein to promote lymphangiogenesis and reduce edema, thereby preserving heart functions after MI.

2.4. MI Therapy Performance of Hep@VEGF-A Complexes

Angiogenesis after MI is gaining attention in improving repairment.[16c,22,23] VEGF-A is an important pro-angiogenic factor.[6] Hep@VEGF-A was administered 5 d post-MI as angiogenesis typically occurs after an early stage inflammatory response period (3–4 d post-MI).[1,24] Relevant heart functions were detected (Figure 5c) and angiogenesis was analyzed by immunohistochemical analysis of CD31 (Figure S5b, Supporting Information). Our results indicated that the treatment with Hep@VEGF-A complexes promoted the recovery of heart functions through promotion of angiogenesis. However, the scar area showed no significant reduction (Figure 6a,d,b,e), which is potentially attributable to the strong vascular permeability of newly formed vessels.[7a,9b]

2.5. Coordinated MI Therapy of Hep@VEGF-C and Hep@VEGF-A Complexes

The repair of MI is a continuous process.[24] Restoring collateral circulation is vital to re-establish the nutrition and oxygen supply to the ischemic area, thus promoting myocardial survival.[16] The newly formed blood vessels increase the interstitial edema owing to the vascular permeability.[9a–c] Thus, it is ideal to promote lymphatic vessels to alleviate cardiac edema and to coordinately stimulate angiogenesis to restore the myocardial blood supply (Scheme 1 and Figures 5 and 6).

To investigate the appropriate relative proportions of VEGF-C to VEGF-A for MI therapy, HUVECs were treated with various ratios [6:1, 3:1 (6:2), 3:2 (6:4), and 1:1 (6:6)] with a fixed VEGF-C content. Tube formation was observed 8 h after the treatment.[22] The observed tube formation improved with an initial increase in VEGF-A percentage. However, there was no observed increase in the number of vessels when VEGF-A was applied at ratios beyond 3:1 (Figure S7, Supporting Information). These results suggest a specific ratio of VEGF-C to VEGF-A (VEGF-C/A) will exert a maximal therapeutic effect.
Based on the above results, a coordinated MI therapy composed of functional complexes was performed at the dose proportions of 1:1 (Hep@VEGF-C vs Hep@VEGF-A, termed as Hep@VEGF-C/A(1:1)) and 3:1 (Hep@VEGF-C vs Hep@VEGF-A, termed as Hep@VEGF-C/A(3:1)) (Figure 5a). Cardiac functions were evaluated by echocardiography 4 weeks post-MI. In vivo fluorescence images show the newly formed lymphatic vessels (stained with Prox-1 or VEGFR-3, pointed by the arrow) in mouse heart tissues 1 and 3 d post-MI (n = 3 per group; Data: Mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001).

Figure 4. In vivo fluorescence images show the newly formed lymphatic vessels (stained with Prox-1 or VEGFR-3, pointed by the arrow) in mouse heart tissues 1 and 3 d post-MI (n = 3 per group; Data: Mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001).
post-MI (Figure 5c). The mice were then sacrificed. The HW/BW, HW/TL (Figure 5b), cardiac fibrosis area (Figure 6a,d,b,e), and changes in compensatory hypertrophy of myocardial cells (Figure 6c,f) were investigated. Compared with the control group, the coordinated treatment with Hep@VEGF-C/A(1:1) significantly promoted an improved MI repair, yet it did not achieve better results than the Hep@VEGF-C treatment group. Compared to the Hep@VEGF-C group, the coordinated Hep@VEGF-C/A(3:1) therapy improved heart functions and reduced the fiber scar area more comprehensively after MI.

VEGF-C (0.5–1 h post-MI) and VEGF-A (5 d post-MI) were administered during different stages of MI via HepNP carriers to promote lymphangiogenesis and angiogenesis, respectively. The new capillary walls in the second stage induced by Hep@VEGF-A have high permeability, which increase cardiac edema.[7c,9d] Lymphangiogenesis induced by the Hep@VEGF-C in the first stage could eliminate the exudate. However, when the amount of administered Hep@VEGF-A in the second stage (5 d post-MI) was too high (Hep@VEGF-C/A(1:1)), the exudate from the newly formed vessels were too much to be eliminated by the newly formed lymphatic vessels, preventing the coordinated treatment from achieving additional benefits. Thus, the second stage of stimulating angiogenesis by Hep@VEGF-A must be maintained within a specific range.
to ensure the elimination of exudate by lymphangiogenesis during the first stage.

Based on the above results, it could be inferred that the HepNP vector was efficient in delivering VEGF proteins as cardiovascular diseases therapy. Toxic injury caused by Hep@VEGF complexes in other organs also present a concern. Therefore, the morphological changes of liver, lung, kidney, and spleen were investigated by hematoxylin-eosin (H&E) staining (Figure S8, Supporting Information). No obvious pathological abnormalities were observed, thereby further confirming the safety of the HepNP vector and demonstrating its potential for protein delivery.

3. Conclusions

Herein, crosslinked negatively charged heparin polysaccharide-based nanoparticle named HepNP was successfully used for protein delivery. HepNP can efficiently condense VEGF proteins due to its unique electronegative sulfonic acid and carboxyl domain. HepNP delivered VEGF-C (lymphangiogenesis promoter) and VEGF-A (angiogenesis promoter) proteins into cardiac tissue to restore the heart functions of mice with MI. We also developed a strategy using HepNP to coordinately deliver VEGF-C and VEGF-A proteins into hearts, effectively promoting collateral circulation, reducing edema, and improving

Figure 6. Coordinated protein therapy for MI with the sequential administration of Hep@VEGF-C (0.5–1 h post-MI) and Hep@VEGF-A (5 d post-MI) (Hep@VEGF-C vs Hep@VEGF-A, termed as Hep@VEGF-C/A). a,d,b,e) Representative images and quantification analysis of Red Sirius and Masson trichrome stained heart sections. c,f) Representative images and quantification analysis of WGA stained myocytes (sham n = 3; control n = 4; Hep@VEGF-C n = 6; Hep@VEGF-A n = 4; Hep@VEGF-C/A(1:1) n = 4; Hep@VEGF-C/A(3:1) n = 6; WGA: Wheat germ agglutinin; Data: Mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001).
cardiac function at the dose ratio of 3:1 (Hep@VEGF-C vs Hep@VEGF-A). This strategy reduced the dosage of protein drugs and the Hep@VEGF delivery system was administered intravenously; this delivery method was found to be more economical and convenient for the treatment of cardiovascular diseases. Furthermore, due to the unique binding ability of heparin with cytokines and growth factors, HepNP also displays great potential for application in therapy of other serious diseases. Mechanistic studies, as well as studies on further protein-delivery applications of HepNP carrier, are ongoing and will be reported in due course.

4. Experimental Section

Preparation of HepNP and Loading of Protein: According to the reported preparation method, heparin nanoparticle (named HepNP) was synthesized for protein delivery by modifying lipico acid onto heparin (Hep-LA). The disulfide bonds in Hep-LA were broken and recrosslinked to get crosslinked HepNP. Zetasizer Nano ZS90 and AFM were then used to characterize the HepNP. VEGF-C protein was chosen as the model protein for the characterization of heparin based carrier (HepNP). The HepNP and VEGF-C were first mixed and assembled at different weight ratios (W_{HepNP}:W_{VEGF-C}: 50:6 or 100:6) to obtain the functional complexes Hep@VEGF-C. Heparin macromolecule was also mixed with VEGF-C (at the weight ratio of 100:6) for characterization of VEGF-A containing delivery systems. The obtained delivery systems were characterized by Zetasizer Nano ZS90 and AFM.

Binding Affinity Assay: Binding affinity of VEGF-C with cells delivered with or without HepNP carrier was further investigated. HUVECs were seeded into the plate at a density of 80% and cultured for 24 h. VEGF-C protein labeled with the fluorescent molecule Cy7-SE was then assembled with the carrier. The obtained complexes were added to the plate at a protein content of 0.2 µg per cell. After cultured for 8 h, the medium was gently removed, and the nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). The binding situations of the complexes were observed with CLSM.

To further investigate the release of VEGF-C or VEGF-A protein from the HepNP-based delivery system, the protein and HepNP with Cy7-SE and FITC, respectively, were labeled. HUVECs were then seeded into the plate as has been described and cultured for 24 h. VEGF-C or VEGF-A protein labeled with the fluorescent molecule Cy7-SE was then assembled with the FITC labeled HepNP carrier. The obtained complexes were added to the plate at a protein content of 0.2 µg per cell. After cultured for 4 or 8 h, the medium was gently removed, and the nuclei were stained with DAPI. The release of VEGF protein from HepNP carrier was observed with CLSM.

Immune Responsive Assay: A typical obstacle of protein therapy was the triggering of immune rejection response. WT C57BL/6j male mice of 6–8 weeks old (~25 g) were divided into three groups including sham, VEGF-C, and Hep@VEGF-C. The groups were injected 0.5–1 h after the MI surgery with 100 µl of physiological saline, 2.4 µg of VEGF-C protein and Hep@VEGF-C complex containing equivalent protein via eye canthus iv injection for once, respectively. It should be mentioned that VEGF-C used in this experiment was labeled with Cy7-SE. IVIS Spectrum were used to observe the accumulation and retention of the protein. The corresponding organs were also harvested for further detection. The heart tissues harvested after 72 h of injection were sliced and observed under CLSM to confirm the accumulation and retention.

MI Therapy by Hep@VEGF-C Complexes: A total of 20 WT C57BL/6j male mice of 6–8 weeks old (~25 g) with MI were randomly divided into four groups and treated 0.5–1 h after the MI surgery with 100 µl of physiological saline (control group), 0.6 µg of VEGF-C protein (VEGF-C group), Hep@VEGF-C complexes (0.6 µg of VEGF-C protein, Hep@VEGF-C group), and HepNP (equivalent HepNP with Hep@VEGF-C, HepNP group) via eye canthus iv injection for once. The corresponding heart tissues were collected 1 and 3 d post-MI. As VEGF-C protein mainly influence the formation of lymphatic, immunofluorescence assay was performed to evaluate the lymphangiogenesis. The heart tissues were sliced after frozen, and the obtained slices were first treated with VEGFR-3 or Prox-1 antibody and incubated with Alexa Fluor 555-, 488-conjugated secondary antibody. The slices were finally observed under CLSM. The corresponding images were captured by a Nikon Eclipse TE2000-S microscope (Nikon, Japan), and analyzed by Image Pro Plus 3 (Nikon). The number of newly formed lymphatic vessels were counted with the average value of at least 8 images per heart in a double-blind fashion.[11d] The final purpose to deliver VEGF-C with HepNP was to reduce the edema post-MI, thus, the water content of cardiac was evaluated by wat weight versus dry weight method by desiccating the harvested heart tissue for 2 d at 65 °C.[11d,20]

MI Therapy by Hep@VEGF-A Complexes: A total of 16 WT C57BL/6j male mice of 6–8 weeks old (~25 g) with MI were divided into two groups (control and Hep@VEGF-A) and treated with 100 µl of physiological saline and Hep@VEGF-A complexes (0.6 µg of VEGF-A protein) 5 d post-MI via eye canthus iv injection for once. Hep@VEGF-A was prepared the same way as VEGF-C (W_{HepNP}:W_{VEGF-A}: 100:6). The corresponding heart tissues were collected 4 weeks after MI. As VEGF-A protein mainly influence the angiogenesis, immunohistochemical assay was performed to detect the newly formed vessels. The heart tissues were sliced after frozen, and the obtained slices were treated with CD31 antibody. Images were captured and the new vessels were analyzed as described earlier.[11d] The cardiac function and fibrosis score were also evaluated, and the details were described later.

Coordinated MI Therapy by Hep@VEGF-C and Hep@VEGF-A Complexes: As angiogenesis plays important role in MI therapy for restoring heart function, a coordinated therapy was proposed that VEGF-C protein was delivered in the initial of MI for stimulating lymphangiogenesis to reduce edema and VEGF-A protein was then delivered for angiogenesis to restore heart function.

The tube formation assay was conducted as described previously to detect the suitable dose ratio of VEGF-C vs VEGF-A (termed as VEGF-C/A). The 96-well plate was coated with Matrigel (BD Biosciences) by adding 50 µl of ice-cold Matrigel solution per well and incubated at 37 °C for 1 h. HUVECs were seeded into the Matrigel-coated wells (2 × 10^4 cells per well). The cells were directly treated with phosphate buffered saline (PBS) or different ratios of VEGF-C/A (6:1, 3:1:6:2), 3:2(6:4), 1:1(6:6)). Cells were then incubated in a humidified 5% CO2 incubator at 37 °C for 8 h and washed carefully with PBS. After incubation at 37 °C for an additional 30 min, the cells were washed carefully with PBS and endothelial cell tubes were observed under the Nikon microscope at magnification ×100. The total tube number from the images were quantified using the AngioQuant v1.33 software (MathWorks, Natick, MA) with at least 5 images per well. The experiments were performed at three independent times.[22,24] According to the tube formation, the ratio of VEGF-C and VEGF-A at 1:1 and 3:1 were chosen for the following coordinated therapy. A total of 32 mice with MI were randomly divided into different groups including control, Hep@VEGF-C, Hep@VEGF-C/A(1:1), and Hep@VEGF-C. The groups were injected 0.5–1 h after the MI surgery with 100 µl of physiological saline, 2.4 µg of VEGF-C protein and Hep@VEGF-C complex containing equivalent protein via eye canthus iv injection for once, respectively.
Hep@VEGF-C/A(3:1). In the first stage (0.5–1 h after MI surgery). The control group were treated with physiological saline, while the other three were treated with Hep@VEGF-C (0.6 µg VEGF-C) via eye canthus iv injection for once. 5 d later, the treatment of the second stage was performed as follows, the control and Hep@VEGF-C/A groups were treated with physiological saline, while Hep@VEGF-C/A(1:1) group was treated with Hep@VEGF-A (0.6 µg VEGF-A) and Hep@VEGF-C/A(3:1) group was treated with Hep@VEGF-A (0.2 µg VEGF-A) via eye canthus iv injection for once. It should be mentioned that the normal mice without treatment were the sham group. All the mice were raised in a specific pathogen free environment till 4 weeks after MI. Vvo770 high-resolution microimaging system was used to evaluate the cardiac function of mice from the parasternal short-axis 2D mode. Left ventricular end-systolic volume and wall thickness were measured in M mode. The mice were then harvested and the tissues were observed. The fibrotic area of heart tissue sections was detected by Sirius red and Masson staining. The percent of fibrosis (blue in Masson staining or red in Sirius red staining) was analyzed and calculated by a NIS-ELEMENTS quantitative automatic program (Nikon, Tokyo, Japan). Sections of heart were also stained with WGA to assess cardiomyocyte hypertrophy.[21]

The tissue integrity of liver, kidney, lung, and spleen were also detected by H&E staining at the level of paraffin section to evaluate the in vivo toxicity of HepNP vector.[16]

**Statistical Analysis:** Student’s t-test and one-way ANOVA were used. More details were described in the Supporting Information.

### Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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### Conflict of Interest

The authors declare no conflict of interest.

### Keywords

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