Novel Angiogenesis Therapeutics by Redox Injectable Hydrogel -

Regulation of Local Nitric Oxide Generation for Effective Cardiovascular Therapy

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**Conflicts of interest**

The authors declare that they have no competing financial interests.

**Author contributions**

Y.N. and L.B.V. conceived and designed the project and wrote draft manuscript. L.B.V. synthesized and analyzed the prepared materials, performed *in vitro* and *in vivo* experiments, analyzed and interpreted the results. Q.T.B. performed the MI model mice and analyzed the echocardiogram data. H.S. and Y. H. advised to this project and the MI model mice. T.T. assisted for angiogenesis experiments and live imaging *in vivo.* All authors revised and edited the final manuscript.
Abstract

Nitric oxide (NO) possesses various functions in cardiovascular diseases; however, due to an extremely short half-life and low bioavailability, its therapeutic application is limited. In inflamed tissues, overproduced reactive oxygen species (ROS) rapidly react with the endogenous NO, reducing its bioavailability. Here, we developed a controllable NO-releasing injectable hydrogel (NO-RIG) formed by the electrostatic crosslinking between the polyion complex flower-type micelles composing of functional polymers to scavenge overproduced ROS and regulate the local NO expression level simultaneously. After the intracardiac injection to mice, NO-RIG converted to gel via physiological temperature-responsive character, distributed homogeneously, and retained in the myocardial tissue for more than 10 d. Treatment with NO-RIG remarkably decreased the infarction size and improved the heart function after myocardial infarction when compared to control injectable hydrogels, such as a simple NO-releasing or ROS-scavenging injectable gels. We found that NO-RIG treatment significantly enhanced the angiogenesis and new blood vessels formation in
mice through the regulation of the NO sustained release and redox equilibrium.

NO-RIG presents high potential in preventing and treating cardiovascular diseases.

**Key words:** redox injectable hydrogel, nitric oxide delivery, reactive oxygen species, polyion complex micelle, myocardial infarction

1. Introduction

Although cardiovascular disease is the leading cause of the death worldwide, the development of effective therapies is still challenging\(^1\)\(^-\)\(^3\). NO, an endogenously produced bioactive gas molecule, plays an important role in protecting and regulating cardiovascular functions\(^4,5\). NO is synthesized by many types of cell in the body including endothelial cells, macrophages, and nervous cells, which are highly expressing endothelial NO synthase (eNOS), inducible NO synthase (iNOS) and neuronal NO synthase (nNOS), respectively\(^6,7\). These NOS systems continuously catalyze intracellular L-arginine (L-Arg) to generate equimolar amounts of NO and L-citrulline via a complex oxygen-dependent five-electron-transfer reaction\(^8\). L-Arg is the precursor and modulates the production of nitric oxide in healthy and pathological conditions\(^9,10\). In order to enhance NO generation, the L-Arg supplementation has been suggested to improve the cardiovascular functions; however, the clinical practices
indicated controversial results\textsuperscript{11–13}. L-Arg, a low-molecular-weight amino acid, spreads non-specifically to the entire body and it is rapidly metabolized after administration, leading to low bioavailability at the target site and insufficient efficacy. On the other hand, versatile NO delivery systems have been developed to enable controlled release of NO at the desired target sites for therapies, antibacterial applications, and so on\textsuperscript{14,15}. Since NO is a small gaseous molecule with low bioavailability, once generated at the target site, it rapidly metabolizes.\textsuperscript{16,17} One of the other important issues to maintain the NO level at the target site is avoiding the reaction of NO with reactive oxygen species (ROS).\textsuperscript{18} It is generally known that huge amounts of ROS are generated in diseased tissues, including during cardiovascular diseases\textsuperscript{19,20}. The overproduced ROS rapidly metabolizes NO to form peroxynitrite, which further induces nitrosative stress, tissue injury, and reduces the bioavailability of NO\textsuperscript{3,21,22}. Recently, it has been reported that the oxidative stress or ROS is one of the most important factor to modulate the NO signaling and induce desensitization of the downstream signaling pathway of NO molecules, resulting in NO resistance\textsuperscript{5,23,24}. Several strategies have attempted to improve NO bioavailability by increasing endogenous NO synthesis\textsuperscript{25}, inhibiting the NO metabolism\textsuperscript{26}, and activating NOS systems\textsuperscript{27}. Dietary antioxidant treatments, such as polyphenols, are reported to increase the NO generation due to suppression of
oxidative stress and ROS.\textsuperscript{28} However, none have been reported to effectively control the level of NO and redox equilibrium in regulation of the cardiac functions after myocardial infarction (MI).

We have recently developed an NO-releasing nanoparticle that responses to macrophages in a tumor microenvironment. The nanoparticles are prepared using the polyion complex (PIC) between poly(ethylene glycol)-\textit{b}-poly(L-arginine) (PEG-PArg) block copolymer and chondroitin sulfate\textsuperscript{29}. The nanoparticles are captured by the macrophages accumulating at the tumor site, which is followed by the cleavage of PArg by an endogenous peptidase to liberate the L-Arg monomer. Finally, overexpressed iNOS in the activated macrophage can catalytically convert L-Arg to NO. We clearly showed a dose-dependent effect of our PArg-based nanoparticles on the progression and suppression of tumor\textsuperscript{29}.

In this study, instead of the A-B type diblock copolymer (PEG-PArg), we designed a new injectable gel based on the A-B-A type triblock copolymer (PArg-PEG-PArg, \textbf{Supplementary Figs. 1,2}) coupled with poly(acrylic acid) (PAAc). The obtained PIC micelle is a so-called flower-type micelle because of the loop structure of PEG chain as a shell, and it converts to hydrogel with the elevated temperature at physiological conditions. In myocardial infarction, infiltration of
inflammatory cells such as macrophages and the accompanying inflammatory response are essential for tissue repair, while once it becomes excessive, it will retard wound healing and fall into chronic heart failure\textsuperscript{30,31}. Since these activated macrophages contribute to a significant increase in iNOS expression and activity, NO can be generated by the iNOS enzymatic reaction by supplying L-Arg as a substrate\textsuperscript{32,33}. Therefore, our idea was to apply this PArg-based injectable gels for local treatment of cardiovascular disorders by generating NO through these local enzymatic reactions. Separately, we have developed a redox injectable gel (RIG), which consists of nitroxide radicals containing the A-B-A type triblock copolymer (PMNT-PEG-PMNT, Supplementary Figs. 3,4) and PAAc. RIG has been reported to eliminate ROS at the local injection site in several applications, such as in periodontitis\textsuperscript{34}, tissue anti-adhesive spray\textsuperscript{35}, and local protein delivery\textsuperscript{36}. Here, using both triblock copolymers, PArg-PEG-PArg and PMNT-PEG-PMNT coupled with PAAc, we developed a novel injectable hydrogel (NO-RIG, Fig. 1a) that showed significantly therapeutic potential in cardiovascular disorders by controlling the NO level and redox equilibrium simultaneously at the local injected sites.

2. Materials and Methods
2.1. Synthesis of polymers and preparation of injectable hydrogel

Synthesis of PArg-PEG-PArg. Triblock copolymer PArg-PEG-PArg was synthesized via ring open polymerization (ROP) of Z-protected L-Ornithine-N-carboxyanhydride [Orn(Z)-NCA, Tokyo Chemical Industry Co., Ltd., Tokyo, Japan] followed by guanidinylation with \(N,N'\)-bis(tert-butoxycarbonyl)-1H-pyrazole-1-carboxamidine [PCX(Boc₂), Tokyo Chemical Industry Co., Ltd., Tokyo, Japan], as shown in Supplementary Fig. 1. The synthetic procedure was described in our previous report with modification\(^{26,34}\). Briefly, \(\text{H}_2\text{N-PEG-NH}_2\) (Mw = 10,000; 1 g, 0.1 mmol) was used as initiator for ROP of Orn(Z)-NCA (1 g, 3.42 mmol) to synthesize POrn(Z)-PEG-POrn(Z) followed by deprotection of the Z-group by acid mixture of trifluoric acid (TFA, 7.5 mL; Wako Pure Chemical Industries, Osaka, Japan) and bromic acid (HBr, 2.5 mL; Wako Pure Chemical Industries, Osaka, Japan) to obtain POrn-PEG-POrn. Next, PArg(Boc₂)-PEG-PArg(Boc₂) polymers were synthesized by guanidinylation of POrn-PEG-POrn and subsequent deprotection of the Boc groups by TFA to obtain PArg-PEG-PArg. The obtained polymers were purified by reprecipitation with diethyl ether and dialysis against water. The repeating number of L-Arg was 24, which was determined by \(^1\text{H} \text{NMR measurement (JEOL ECS-400, JEOL Ltd., Tokyo, Japan) as shown in Supplementary Fig. 2.}
Synthesis of PMNT-PEG-PMNT. PMNT-PEG-PMNT triblock copolymer was synthesized as previously reported with modification\(^3\)\(^6\). Briefly, poly(chloromethylstyrene)-PEG-poly(chloromethylstyrene) (PCMS–PEG–PCMS) triblock copolymer was synthesized by RAFT polymerization of CTA-PEG-CTA (chain transfer agent) (Mw = 10,000). To obtain PMNT-PEG-PMNT, the chloromethyl groups on the PCMS segment of the triblock copolymer were converted to nitroxide radicals via the amination of PCMS-PEG-PCMS with 4-amino- TEMPO (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) in dimethyl sulfoxide (DMSO, Wako Pure Chemical Industries, Osaka, Japan), as shown in Supplementary Fig. 3. The obtained polymers were purified by reprecipitation with diethyl ether, followed by freeze drying with benzene. The repeating unit of CMS was 30, and TEMPO modification rate was about 85% as determined by \(^1\)H NMR and ESR measurements, respectively (Supplementary Fig. 4). Control triblock copolymer (PMNOH-PEG-PMNOH) without nitroxide radical was synthesized by amination of PCMS-PEG-PCMS with 2-aminoethanol (Wako Pure Chemical Industries, Osaka, Japan, the \(^1\)H NMR as described in Supplementary Fig. 5).

Preparation of flower-type PIC micelle and gelation experiments. 5 mg/mL of PMNT-PEG-PMNT or PArg-PEG-PArg or PMNOH-PEG-PMNOH triblock copolymer
was added to 5 mg/mL of poly(acrylic acid) (PAAc) in a phosphate buffer (PB) solution (50 mM, pH 6.2) with the molar ratio of cation:anion as 1:1. The PIC size was determined by dynamic light scattering (DLS) using Zetasizer Nano series ZEN3600 (Malvern Instruments Ltd., Worcestershire, UK), as shown in Supplementary Fig. 6a. Next, the PIC solution was concentrated to the desired concentration of cationic polymer (30–60 mg/mL of PMNT-PEG-PMNT or PArg-PEG-PArg), and the gelation test was confirmed at 37 °C within 1 min. Rheological evaluations of the gels were conducted using a rheometer (MCR302, Anton Paar). A parallel plate with 20 mm diameter and a gap of 0.1 mm was used. The PIC flower micelle solution was put between the plates using a micropipette. Rheological properties (storage modulus $G'$, loss modulus $G''$, and complex viscosity) of temperature dependencies from 15 to 40 °C were measured at a fixed frequency of 1 Hz, a heating/cooling rate of 1 °C/min, and strain amplitudes from 0.5% to 15%, which was within the linear viscoelastic regime. The rheological data of tested gels were shown in Supplementary Fig. 6b.

2.2. Evaluation of the toxicity and angiogenesis in vitro

The in vitro toxicity of NO-RIG was evaluated in the RAW 264.7 mouse macrophage cell line (ATCC, Manassas, VA, USA) using a MTT kit (Roche Diagnostics, Tokyo, Japan). The determined concentrations of PIC were added to each well (96-well plate)
containing 5,000 cells, and incubated for 3 d with normal DMEM medium (Wako, Pure Chemical Industries, Ltd., Japan). After 3 d incubation, cell viability was evaluated using MTT agents according to manufacturer’s protocol. To evaluate the angiogenesis, a cool solution of Matrigel (BD, Tokyo, Japan) was added to 96-well plate (10 µL/well) and incubated to achieve gelation. After that, HUVECs (5,000 cells/well; Riken BioResource Center, Ibaraki, Japan) in DMEM without L-Arg (ThermoFisher, Tokyo, Japan) were added on the surface of the matrigel, followed by the addition of L-Arg (100 µg/mL) or PArg-PIC (100 µg/mL) or VEGF (50 µg/mL). At 6 h after incubation, the angiogenesis was evaluated using a confocal microscope and was analyzed by ImageJ (NIH, MD, USA).

2.3. Gel degradation in vitro

A solution of PIC (60 mg/mL, 200 µL) was incubated at 37 °C for 1 min for gelation. After gelation, 300 µL of PBS buffer was added (Supplementary Fig. 7). The gel with PBS was incubated at 37 °C with shaking speed of 100 rpm. At the interval time, the upper solution was taken out and analyzed by ESR or fluorescent measurements, and the fresh PBS was added.

2.4. Animals
All experiments were carried out using 7 to 8-week-old male ICR mice (32–35 g) purchased from Charles River Japan, Inc. (Yokohama, Japan). Mice were maintained in the experimental animal facilities at the University of Tsukuba under controlled temperature (23 ± 1 °C), humidity (50 ± 5%), and lighting (12 h light-dark cycles). The animals were given free access to food and water. All experiments were performed in accordance with the Regulation for Animal Experiments in the University of Tsukuba (Animal experiment approval number 16-322) and the Fundamental Guideline for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science, and Technology.

2.5. Gel retention in vivo. The gel retention in vivo was determined by IVIS system (Caliper Life Science, USA) using Hilyte Fluor 647-conjugated PMNT-PEG-PMNT (Hilyte-PMNT-PEG-PMNT), which was described in our previous report. The mice were anesthetized with isoflurane before intracardiac injection of Hilyte 647-labeled-PMNT-PEG-PMNT or Hilyte 647-labeled-NO-RIG (20 µL; 60 mg/mL). At the predetermined time, the mice were sacrificed to collect the heart for IVIS measurement with the excitation and emission wavelength was 640 nm and 680 nm, respectively. After the IVIS imaging measurements, the hearts were fixed with 4%
formalin for paraffin-block embedment. Then, 5-µm-thickness sections were prepared and the gel distribution in heart tissues was visualized using a confocal fluorescent microscope (Zeiss LSM 700, Carl Zeiss Microscopy GmbH, Jena, Germany).

2.6. Myocardial infarction (MI) model mice and gel treatments

MI model mice were prepared by ligation of the left anterior descending artery (LAD) according to previous reports with slight modifications. Briefly, the 4th intercostal space was delicately dissected and the position of coronary artery was then determined for ligation at 1-2 mm below the tip of the left atrial appendage in its normal position, which induced roughly 50% ischemia of the left ventricle (right below the suture) and the mice were adapted for survival in most cases. For moderate MI model, the thoracotomy was placed in the 5th intercostal space, and the LAD was performed at the middle position of coronary artery. After the position of LAD coronary artery was determined, a 6-0 suture was passed gently underneath the LAD coronary artery, and the needle was placed carefully to avoid entering the cavity of the left ventricle, but at the same time care was taken to not be too superficial, as the suture would cut through the wall of the ventricle. The suture was then tied with four knots and LAD was remained on the surface of left ventricle after 4 weeks until sacrifice for evaluation. The LAD occlusion was confirmed by the change of color (becoming pale) of the left
ventricular anterior wall. Then, 50 µL solution (60 mg/mL) of different PIC micelles were intracardially injected with the single dose at the ligation site. The gelation was confirmed immediately after injection (Fig. 2a). Finally, the curved mosquito forceps were removed, and the chest cavity was closed with one or two 3-0 silk sutures. The muscles and skin were closed layer by layer with 3–0 silk sutures. After the mouse resumed a normal breathing, the endotracheal tube was removed. In 1.5-2 hours after the surgery, the mouse was completely awake and moving around in the cage. Cardiac functions were determined using echocardiogram measurement and infarction size from heart tissue after 4-week treatment was evaluated by Masson Trichrome staining using ImageJ analysis.

2.7. Echocardiogram measurement

To evaluate the cardiac functions after the treatment, echocardiography was performed using Vevo 2100 Imaging System (VisualSonics Inc., Toronto, Canada). Left ventricle internal dimension in diastolic (LVD;d), systolic (LVD;s) and left ventricular ejection fraction (EF) and fractional shortening (FS) were measured with M-mode. Reduced cardiac function, left ventricular dilation and wall thinning were indicators of a successful MI model.

2.8. Histology
One month after treatment, heart tissues were fixed in 4% formalin and embedded with paraffin block. Then, 5 µm sections were prepared and performed with Masson Trichrome staining was performed for evaluation of infarction areas using a light microscope (Olympus model BZ-X710 Keyence, Tokyo, Japan). For angiogenesis evaluation, skin tissue sections were stained with Hematoxylin and Eosin (H&E) and the number of blood vessels were also analyzed using a light microscope.

2.9. Live imaging for angiogenesis evaluation and NO detection in vivo

The hair at abdomen was removed from the mice and 50 µL of test PIC solution (60 mg/mL) with or without LPS (1µg/mL) was intradermally injected into the abdominal skin of mice (Fig. 3a). At day 7 after the injection, the peritoneal membrane was expanded upside down to visualize the blood vessels (Fig. 3a). Live imaging was recorded under a light microscope (Leica, Tokyo, Japan) to observe the angiogenesis at the injection sites. The density of blood vessels was determined by following formula using Image J (NIH, MD, USA) software. Blood vessel density = (vessel area/total area) × 100% (three different areas per injected site), and the value of density of blood vessel in the sham gel group was normalized to 100%. After imaging of the angiogenesis, 150 µL of diaminofluorescein-FM diacetate (DAF-FM DA, 50 µM, Goryo Chemical, Inc., Tokyo, Japan), a fluorescent probe for the detection and bioimaging of NO, was added to the backside of skin and incubated for 1 h, followed by live imaging with a confocal fluorescent microscope (Zeiss LSM 700, Carl Zeiss Microscopy GmbH, Jena, Germany), using an excitation wavelength 488 nm and emission wavelength 515 nm.

2.10. Statistical analysis
All values are expressed as mean ± standard error of the mean (SEM). Differences between groups were examined for statistical significance using Student’s t-test or one-way analysis of variance, followed by Turkey’s post hoc test (SPSS software; IBM Corp, Armonk, NY). A value of P < 0.05 was considered significant for all statistical analyses.

3. Results and Discussions

3.1. Preparation and Characterization of NO-RIG

NO-RIG was prepared from PIC flower-type micelles, which are composed of the following two biofunctional polycationic polymers: 1) PArg-PEG-PArg (NO releasing polymer) and 2) PMNT-PEG-PMNT (ROS scavenging polymer), in a complex with polyanion PAAc (Fig. 1a). The syntheses and characterizations of PArg-PEG-PArg and PMNT-PEG-PMNT are described in detail in Supplementary Figs. 1-4. The obtained PIC micelles (NO-RIG) were transparent in solution below 25 °C and then converted to gel under physiological conditions at 37 °C (Fig. 1a); thus, we can utilize this PIC system as an injectable hydrogel. The size of PIC micelles was approximately 90 – 100 nm in solution at room temperature (Fig. 1b). By enhancing the ionic strength and temperature, the viscosity of the PIC solution dramatically increased by four orders of
magnitude (Fig. 1c), and the sol-gel transition temperature (where storage modulus (G’) and loss modulus (G’”) became the same G’ = G’”) occurred at around 30 °C due to the destabilization of PIC flower micelles via increased thermal motion energy (Fig. 1d), and both modulus (G’ and G’”) did not change with decreasing temperature, indicating irreversible gel formation (Fig. 1d). This is sharp contrast to the conventional A-B-A-type triblock copolymers, possessing the hydrophobic A-segments and the hydrophilic B-segment, which form temperature-responsive reversible gel due to the hydrophobicity-induced aggregation. In the NO-RIG system, a partial disintegration of the PIC core due to the elevated ionic strength might contribute the irreversible crosslinking between the micelles via the electrostatic crosslinking (Fig. 1a,d). In vitro disintegration of the gel was monitored in phosphate buffered saline (PBS) at pH 7.4 and phosphate buffer (150 mM) at pH 6.2, which mimics the pH in the inflamed or infarcted regions of tissue. Since the gel was formed by physical crosslinking, the gels slowly disintegrated to release the polymer for up to more than 10 d, regardless of the composition and pH, as shown in Supplementary Figs. 7-8a. Because the nitroxide radicals in the PMNT segments show typical triplet spectral in the electron spin resonance (ESR) measurements, supernatant solution was monitored by the ESR and confirmed the release of polymer signals from NO-RIG (Supplementary Fig. 7).
to long retention and gradual disintegration of NO-RIG, it is anticipated to sustainably deliver NO and scavenge ROS in diseased tissues.

We next evaluated the cytotoxicity of PICs in the *in vitro* using the RAW 264.7 mouse macrophage cell line. As shown in Supplementary Fig. 8b, the PArg-PEG-PArg/PAAc PIC (NO-IG) exhibited significantly lower toxicity in RAW 264.7 macrophage cells as compared to PArg-PEG-PArg polymer only, due to the formation of PIC. It is interesting to notice that PICs composed of PArg-PEG-PArg and PMNT-PEG-PMNT (NO-RIG) showed much lower toxicity in RAW 264.7 cells, which is probably due to the ROS scavenging capacity of PMNT-PEG-PMNT (Supplementary Fig. 8b). The induction of angiogenesis *in vitro* was confirmed using the matrigel angiogenesis assay on human umbilical vein endothelial cells (HUVECs). Although clear statistically significant differences were not observed, we confirmed the increased angiogenesis tendencies of the L-Arg monomer and PArg-based injectable gels (Supplementary Fig. 9).

### 3.2. The efficacy of NO-RIG in cardiovascular disease model mice

We wanted to utilize PICs as an injectable hydrogel for MI mice; therefore, we confirmed the retention and localization of the gel in the heart tissue after intracardiac
injection. When the mice were intracardially injected with the triblock copolymer (Hilyte-labeled PMNT-PEG-PMNT), which does not form a gel under the physiological conditions, the retention of the polymer was only 2 d, and had completely disappeared by 3 d (Fig. 2a,b). It should be noted that the fluorescent intensity of the polymer significantly decreased just 2 h after injection (Fig. 2a,b). On the contrary, the PIC (NO-RIG) solution converted to gel straight after the intracardiac injection, and fluorescent signal remained at the injected site for an extended period of more than 10 d (Fig. 2a,b). We also confirmed the homogeneous distribution of PIC in myocardial tissues after injection (Fig. 2c,d). These results suggested that long retention and homogenous distribution of PIC in myocardial tissues may prolong the therapeutic effect to the MI mice.

To investigate the therapeutic effect of NO-RIG in MI, we utilized the ligation of the left anterior descending artery (LAD) model in mice (Fig. 3a). In order to investigate the NO and ROS scavenging effects, we performed an intracardiac injection of NO-RIG and several control gels, as shown in Fig. 3a,b. During 1 month of MI and gel treatments, we evaluated the cardiac functions using an echocardiogram (Vevo 2100 Imaging System) (Fig. 3c). The left ventricular dilation and wall thinning was observed in MI mice, indicating the induction of infraction and myocardiocyte death 1 month
after LAD ligation (Fig. 3c). When we injected several kinds of control gels such as sham gel (no NO generation and no ROS scavenging), NO-IG (NO generation but not ROS scavenging), and RIG (no NO generation but ROS scavenging), these gels did not show any statistically significant improvements. Only the mice treated with NO-RIG showed a statistically significant recovery of the thickness of myocardial tissues (Fig. 3c,d). In addition, the ejection fraction (EF) and fraction shortening (FS), which are important parameters of cardiac function, were also remarkably improved in the mice treated with NO-RIG compared the other groups (Fig. 3e). After surgery and the gross observation of the heart, we found that all treated groups exhibited a large infarction in the left ventricle (Fig. 4a); thus, we further measured the infarction size by Masson Trichrome staining. As shown in Fig. 4b,c, the infarction size of the mice treated with NO-RIG was significantly smaller than in the other treated groups, although the infarction size was still relatively large (about 40%) due to the high severity of this infarction model. Alternatively, we repeated the experiment using a moderate MI model to confirm the therapeutic efficacy of NO-RIG in this moderate model (Supplementary Fig. 10a). The results showed that the moderate MI mice treated with NO-RIG had a remarkable improvement in cardiac functions and a reduced the infarction size (Supplementary Fig. 10b-e). The obtained results indicate that NO-RIG treatment can
effectively protect the mice from MI when compared to other control injectable hydrogels.

3.3. Investigation the efficacy of NO-RIG in angiogenesis and NO generation in vivo

To investigate the therapeutic mechanism by which NO-RIG protects the heart functions after MI, we assessed the induction of angiogenesis by NO-RIG treatment in mouse abdominal skin, in that we could also evaluate the NO generation in living mice. The NO-RIG and other control gels were injected intradermally into the abdomen, and the angiogenesis was confirmed at day 7 after injection (Fig. 5a). To mimic the condition of MI and to activate the immune cells such as macrophages, we used lipopolysaccharide (LPS, Sigma-Aldrich, St. Louis, MO), which was loaded into the injectable gels. In fact, we have previously confirmed the NO generation in RAW264.7 macrophage cells incubated with PArg-based PIC micelle in the presence of LPS\textsuperscript{29}. We found that the sham gel and PArg-PIC (NO-IG) treatments did not significantly induce the angiogenesis at all, although a little angiogenesis was observed in mice treated with the LPS-loaded control gel (Fig. 5b,c). Interestingly, NO-RIG with LPS treatment clearly induced the angiogenesis in mice, which was significantly higher than in the other treatments (Fig. 5b,c). Alternatively, it was confirmed that the mice treated with
NO-RIG and LPS exhibited a significantly increased production of NO by fluorescent staining of the NO probe with DAF-FM DA (Fig. 6a,b). These results clearly indicate that treatment with NO-RIG can effectively control the NO generation to the desired level in local areas of the diseased tissue, and induces angiogenesis; however, NO-IG did not show any effect. Furthermore, we could also observe the NO generation in nerve fibers (Supplementary Fig. 11), suggesting the partial contribution of nNOS to the generation of NO. Consequently, the number of new blood vessels was significantly higher in the mice treated with NO-RIG and LPS when compared to other controls (Fig. 6c,d).

As stated above, we have already shown that intravenous administration of low dose PEG-PArg-based PIC micelle induces the NO generation and enhances the tumor progression, indicating the angiogenesis inducing effect of poly(L-arginine)\textsuperscript{29}. Since ROS is well-known to overgenerate in diseased tissues, we have previously investigated ROS scavenging capacity of our polymer PMNT-PEG-PMNT. In particularly, PMNT-PEG-PMNT containing hydrogel significantly suppressed superoxide level at peritoneal membrane in talc-induced adhesion mice\textsuperscript{35}. In the other studies, the PEG-PMNT polymer nanoparticle was also previously reported to catalytically scavenge ROS \textit{in vitro} and in several murine disease models, such as renal injury,
cerebral damage, and tumors\textsuperscript{40–43}. It should be noted that these designed polymers exhibited very low toxicity when compared to conventional polymers\textsuperscript{44,45}. Based on the results obtained from this study, we propose an action mechanism of our newly designed NO-RIG, as shown in Fig. 7. Basically, the NO generation is strongly dependent on NO synthases and nitrate-nitrite-NO pathway. In the heart tissue, NO is generally produced when extracellular L-Arg binds to catalytic haem of eNOS which is constitutively expressed in endothelial cells\textsuperscript{5}. Under pathological conditions such as inflammation and oxidative stress, eNOS uncoupling occurs, resulting in oxidation of L-Arg to increase in superoxide anion rather than produce NO\textsuperscript{24,46}. On the other hand, the important source of iNOS is also produced mainly in macrophages and to lesser extent in other cell types such as fibroblast, smooth muscle cells, and even accumulating monocytes in infarcted areas. However, the NO generation and physiological NO signaling can be interrupted by excess ROS at diseased tissues whereas superoxide rapidly interacts with NO to form peroxynitrite\textsuperscript{23,46}. The designed NO-RIG is composed of two biofuntional polymers: 1) PArg-PEG-PArg, which can generate NO by the activated macrophage and other cells in inflamed tissues, and 2) PMNT-PEG-PMNT, which can scavenge overproduced ROS to maintain NO levels in the inflamed areas. Utilizing NO-RIG, we could control the NO level and redox
equilibrium balance at the diseased tissues, which significantly improved the generation of NO and its bioavailability, regardless of endogenous NO generation. As the results, NO-RIG treatment significantly inhibited the progression of MI and improved cardiac functions by stimulating the angiogenesis via regulated NO delivery. The findings in this study showed that the critical relationship of redox balance with NO generation that could be controlled using our NO-RIG system, which further regulates the angiogenesis and cardiac functions after MI.

4. Conclusion

Taken together, our designed NO-RIG, composed of dual biofunctional triblock polymers, is able to maintain the release of NO and improve its bioavailability coupled with ROS scavenging ability. Unlike other conventional drug delivery systems, the use of NO-RIG itself exhibited the potential therapeutic efficiency without any conventional drugs or biomolecules; thus, to further improve the therapeutic efficacy, because NO-RIG is an injectable hydrogel system, versatile drugs and biomolecules or even cells, can be also entrapped in NO-RIG, which it is anticipated to have a high therapeutic efficacy for the treatment of cardiovascular diseases.
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**Figure Legends:**

**Figure 1.** The schematic illustration and characterization of nitric oxide (NO) releasing/reactive oxygen species (ROS) scavenging injectable hydrogel.
(NO-RIG). (a) Schematic illustration of flower-type polyion complex (PIC) micelle composed of PArg-PEG-PArg and PMNT-PEG-PMNT with PAAc. As in the design, the triblock copolymer PArg-PEG-PArg generates NO by locally activated macrophages, and triblock copolymer PMNT-PEG-PMNT scavenges overproduced ROS at injury sites. Gelation mechanism of PIC micelle at 37 °C due to partially destabilization of PIC micelles forming crosslinking. (b) DLS measurement of PIC (5 mg/mL) in PBS 7.4 at 25 °C. (c) Temperature dependence of the complex viscosity of PIC (45 mg/mL, 300 mM ion concentration). (d) Change in storage modulus $G'$ and loss modulus $G''$ of PIC (45 mg/mL) when changing the temperature.
Figure 2. The retention of NO-RIG after intracardiac injection was determined by IVIS imaging measurements using a Hylite-labeled polymer. (a and b) The data are expressed as mean ± SEM, ** $P < 0.01$, $n = 3$. (c and d) The confocal fluorescent microscope imaging of heart sections (5-µm thickness) at day 3 after injection of NO-RIG. (c) Low magnification with scale bars = 100 µm. (d) High magnification with scale bars = 20 µm: surface tissue region (upper panels) and approximately 300-µm deeper region from surface tissues (low panels).
Figure 3. The therapeutic efficacy of NO-RIG in myocardial infarction (MI) mice. (a) LAD ligation model and intracardiac injection of NO-RIG and control gels. (b) The polymeric hydrogels used in the experiment. The polymer compositions and characterizations were shown in Supplementary Figs. 1-5. After 4 weeks of treatment, the cardiac functions were evaluated using Vevo 2100 Imaging System. (c) M-mode echocardiogram imaging of mice 4 weeks after MI and treatment with gels. Scale bar = 2 mm. (d) Interventricular septum thickness at end-diastole (IVS;d) and interventricular septum thickness at end-systole (IVS;s). (e) Ejection fraction (EF) and fractional
shortening (FS) were measured with M-mode. The data are expressed as mean ± SEM, * $P < 0.05$, $n = 6-7$.

Figure 4. Treatment of NO-RIG suppresses infarction after MI. (a) The gross observation of the heart after 4-weeks of treatment. Scale bars = 5 mm. (b) Masson Trichrome staining of 5-µm-thick sections of heart tissue. Scale bars = 500 µm. (c) Infarction size was determined using ImageJ software. The data are expressed as mean ± SEM, * $P < 0.05$, $n = 6–7$. 
**Figure 5.** Treatment of NO-RIG induces angiogenesis after intradermal injection. (a) Intradermal injection of the PICs (60 mg/mL) with and without LPS (1 µg/mL) in the abdominal skin to confirm the angiogenesis and NO generation. (b) Abdominal skin (backside) of the mice after 7 d treatment with PICs (with and without LPS) in living mice. Scale bars = 200 µm (left panels) and = 50 µm (right panels). (c) Quantitative data of the angiogenesis at the injected sites using ImageJ software. The value of density of blood vessel in the sham gel group was normalized to 100%. The data are expressed as mean ± SEM, * $P < 0.05$, ** $P < 0.01$, $n = 3–4$. 
Figure 6. NO generation by NO-RIG treatment induces blood vessel formation. The analysis was performed at day 7 after NO-RIG treatment. (a) Confocal fluorescent microscope imaging for detection of NO generation using DAF-FM DA in the abdominal skin (backside) of living mice. Scale bars = 50 µm. (b) Quantitative data of fluorescent intensity for NO releasing at the injection sites. (c) H&E staining of the abdominal skin at the gel injected sites. Scale bars = 100 µm. (d) Number of blood vessels near the gel injected sites. Yellow arrows indicate the blood vessels. The data are expressed as mean ± SEM, * P < 0.05, ** P < 0.01, n = 3–4.
Figure 7. The investigated and proposed mechanism of NO-RIG in inhibiting MI in mice. NO-RIG is composed of two biofuntional polymers: PArg-PEG-PArg, which generates NO by the activated macrophages in inflamed tissues, and PMNT-PEG-PMNT, which scavenges ROS to enhance NO activity. NO-RIG can sustainably control the NO delivery and redox equilibrium balance in the infarcted tissues. As the results, NO-RIG treatment significantly prevents the progression of MI and improve cardiac functions. SMC: smooth muscle cell; EC: endothelial cell.
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