Published in final edited form as:

ACS Appl Mater Interfaces. 2018 November 07; 10(44): 37783–37796. doi:10.1021/acsami.8b09757.

NIPAM-based Microgel Microenvironment Regulates the Therapeutic Function of Cardiac Stromal Cells

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Abstract

To tune the chemical, physical, and mechanical microenvironment for cardiac stromal cells to treat acute myocardial infarction (MI), we prepared a series of thermally responsive microgels with different surface charges (positive, negative, and neutral) and different degrees of hydrophilicity, as well as functional groups (carboxyl, hydroxyl, amino, and methyl). These microgels were used as injectable hydrogels to create an optimized microenvironment for cardiac stromal cells (CSCs). Our results indicated that a hydrophilic and negatively charged microenvironment created from poly(*N*-isopropylacrylamide-*co*-itaconic acid) was favorable for maintaining high viability of CSCs, promoting CSC proliferation and facilitating the formation of CSC spheroids. A large number of growth factors, such as vascular endothelial growth factor (VEGF), insulin-like growth factor I (IGF-1), and stromal-derived factor-1 (SDF-1) were released from the spheroids, promoting neonatal rat cardiomyocyte activation and survival. After injecting the poly(*N*-isopropylacrylamide-*co*-itaconic acid) microgel into mice, we examined their acute inflammation

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○Author Contributions

X.C. and J.T. contributed equally to this work.

The authors declare no competing financial interest.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.8b09757.

Experiment materials and material characterization protocols, LIVE/DEAD cell images protocol, Figure S1 hydrogel thermal gelation, Figure S2 flow cytometry for hCSCs surface mark, Figure S3 hCSCs culture in microgels at different time points, and Figure S4 NRCM culture in microgels at different time points (PDF)

and T-cell immune reactions. The microgel itself did not elicit obvious immune response. We then injected the same microgel-encapsulated with CSCs into MI mice. The result revealed the treatment-promoted MI heart repair through angiogenesis and inhibition of apoptosis with an improved cell retention rate. This study will open a door for tailoring poly(*N*-isopropylacrylamide)-based microgel as a delivery vehicle for CSC therapy.

Graphical Abstract

Keywords

injectable hydrogels; NIPAM microgel; thermo-responsive microgel; cardiac stromal cells; multicellular spheroids; heart repair

INTRODUCTION

More than 7 million people suffer from myocardial infarction (MI) each year, including segment (ST)-elevation/nonST-elevation myocardial infarctions.¹ Catheter-based intervention^{2–4} has reduced the mortality of MI patients, but the therapeutic effect is limited by the strict time window available after the infarct. Pharmacological reperfusion therapy has been employed to relieve symptoms and improve long-term prognosis for survivors, but cannot prevent the progress of MI evolving into heart failure. Thus, MI treatment still remains a challenge and more effective chronic approaches are still being pursued.⁵ Over the past 15 years, numerous animal and clinical studies have been performed to discern the ability of various stem cells to preserve heart function. However, the effectiveness of stem cell therapy in patients is hampered by fundamental issues, such as extremely poor cell retention in the heart and low transplanted cell survival rates.⁶

Injectable hydrogels have been explored for MI treatment, based on their ability to promote stem cell functions or on their own regenerative properties.⁷ Natural polymers, such as fibrin,⁸ collagen,^{9,10} Matrigel,^{7,10} chitosan,^{11,12} keratin,¹³ and hyaluronic acid,^{14,15} and decellularized myocardial matrix, have been tested and proven to improve heart functions

after MI.^{16,17} However, drawbacks of natural polymer hydrogels, such as poor handling characteristics, inflexibility in design, and their inability to undergo rapid gelation keep them from successful clinical translation.¹⁸ Synthetic polymers, on the other hand, offer a number of advantages. Diverse specialized synthetic polymers, such as poly(lactic-*co*-glycolic acid)¹⁹ and poly(*N*-isopropylacrylamide) (P(NIPAM)),^{20–22} can be incorporated into synthetic hydrogels to facilitate cell growth, enhance gene expression, and improve biological functions.^{23–25} These synthetic variants have been developed for tissue engineering applications, as they can mimic the natural extracellular microenvironment.^{26–28} For example, a hydrogel consisting of poly(*N*-isopropylacrylamide-*co*-acrylic acid) (P-(NIPAM-AA)) and hydroxyethyl methacrylate–poly(trimethylene carbonate) (HEMAPTMC) has been used to treat chronic infarcted myocardium.²⁹ However, the impact that thermally sensitive hydrogels have on the viability, growth, and biological functions of cardiac stromal cells and cardiomyocytes still remains elusive. Previous studies have indicated that the properties of the cellular microenvironment created within scaffolds, including surface charge, microstructure, chemical groups, and hydrophobicity/hydrophilicity, significantly affect cell attachment, growth, migration, differentiation, and biological functions.^{20,21,30–32}

Herein, we explored P(NIPAM)-based synthetic thermos-sensitive injectable microgels (Figure 1a), including poly(*N*-isopropylacrylamide-*co*-itaconic acid) (P(NIPAM-IA)), poly(*N*-isopropylacrylamide-*co*-2-hydroxyethyl methacrylate) (P-(NIPAM-HEMA)), poly(*N*-isopropylacrylamide-*co*-dimethylamino ethyl methacrylate) (P(NIPAM-DMAEMA)), and poly(*N*-isopropylacrylamide-*co*-poly(ethylene glycol) methyl ether acrylate) (P(NIPAM-PEGA)) for culturing human cardiac stromal cells (hCSCs) (also known as cardiosphere-derived cells) and neonatal cardiomyocytes (NRCMs). We examined the effects of the hydrogel microenvironment on CSC attachment, proliferation, growth-factor release, and long-term viability of myocytes. Furthermore, the therapeutic effects of P(NIPAM-IA)-encapsulated hCSCs were investigated in a mouse model of MI.

RESULTS AND DISCUSSION

Characterization of NIPAM Microgels

A schematic design of the study is shown in Figure 1, including the chemical formula (Figure 1a) of P(NIPAM-IA), P(NIPAM-HEMA), P(NIPAM-DMAEMA), and P(NIPAM-PEGA), the in vitro experimental setup used to test the effects of the hydrogel microenvironment on CSC properties (Figure 1b–g), and the in vivo experimental setup used to demonstrate the therapeutic effects in the MI mouse model (Figure 1h). The properties investigated in the in vitro assays were cellular attachment, proliferation, growth-factor release, and the long-term viability of myocytes.

Free radical emulsion polymerization was utilized to synthesize P(NIPAM)-based microgels, including P(NIPAM-IA), P(NIPAM-DMAEMA), P(NIPAM-HEMA), and P-(NIPAM-PEGA). Fourier transform infrared spectroscopy (FTIR) was employed to identify the functional groups of synthesized microgels (Figure 2a). C=O asymmetric stretching and C–N bending within *N*-isopropylacrylamide are shown at 1640 and 1550 cm^{–1}, respectively.³³ The methyl groups of NIPAM associated with C–C stretching and bending as well as

asymmetric C–H bonding are assigned to 1450 cm^{-1} . The C=O bond in the carboxylic group of itaconic acid (IA) is $1710\text{--}1720\text{ cm}^{-1}$. The hydroxyl group from 2-hydroxyethyl methacrylate (HEMA) is associated with the peak at 3440 cm^{-1} .³⁴ The C–O stretching bands in the ether group from poly(ethylene glycol) methyl ether acrylate (PEGA) are assigned to 1050 cm^{-1} .³⁵ The C=O bond (around 1640 cm^{-1}) and C–N bond (around 1566 cm^{-1}) in 2-(*N,N*-dimethylamino)ethyl methacrylate (DMAEMA)³⁶ are overlapped with those from P(NIPAM). The FTIR results confirm the successful preparation of various copolymer microgels. All the microgels were further assessed through ζ -potential measurements, which indicate the surface charge. The ζ -potential for each microgel is shown in Table 1. The incorporated co-monomers influence the ζ -potential of the synthetic microgels. The partial deprotonation of the carboxyl group in P(NIPAM-IA) raises the negative surface charge of the microgel. Apart from the carboxyl group contribution, the KPS initiator also results in the negative surface charge of the synthetic microgels, and this is the reason for the slightly negative charge of both P(NIPAM-PEGA) and P(NIPAM-HEMA) microgels. The amino group in the P(NIPAM-DMAEMA) and the positively charged initiator contribute to the positive surface charge of this microgel. Dynamic light scattering was applied to obtain the hydrodynamic diameters (d_h) of microgels at a concentration of 0.1 mg/mL at various temperatures in phosphate-buffered saline (PBS) buffer. The hydrodynamic diameters are shown in Figure 2b. The d_h of all microgels shrinks with an increase in temperature because of the presence of the NIPAM moiety. All microgels have a considerable shift of d_h around $30\text{--}35\text{ }^\circ\text{C}$. This critical transition temperature is referred to as the volume phase transition temperature (VPTT) of microgels, which is correspondent to the lower critical solution temperature of the linear polymer, NIPAM. To further illustrate the phase transition behaviors as a function of temperature, the plot of the shrinkage ratio, $d_h(T)/d_h(25\text{ }^\circ\text{C})$, versus temperature is shown in Figure 2c. The P(NIPAM-DMAEMA) has the largest shrinkage ratio, while P(NIPAM-IA) shows the lowest shrinkage ratio in comparison with P(NIPAM-HEMA) and P(NIPAM-PEGA), due to a greater amount of hydrophilic carboxylic groups. Scanning electron microscopy (SEM) was employed to characterize the microstructure and morphology of the synthetic microgels' scaffold network. Rapid freezing in liquid nitrogen was used to maximally preserve the original microgel scaffold structure.³⁷ SEM reveals different morphologies and structures for the four microgel scaffolds (Figure 2d–g). P(NIPAM-IA) has the largest pore size, around $50\text{ }\mu\text{m}$, because of its high electrostatic repulsion force, associated with the partially deprotonated carboxyl groups. Such a large pore size may allow cell migration inside the network to promote cell–cell interactions. P(NIPAM-HEMA), on the other hand, has the smallest pore size, around $10\text{ }\mu\text{m}$, due to its strong hydrophobicity. Overall, all hydrogels present a porous network structure that allows nutrients, oxygen, and wastes to move into/out of the cells inside the microgels.

All the microgel dispersions at 30 mg/mL (in physiological saline, at a pH of 7.2) change from a solid to a gel state once the temperature is over $37\text{ }^\circ\text{C}$, and return to the solid state when the gel cools down to room temperature (Figure S1, Supporting Information). The balance of hydrophobic interactions and electrostatic repulsions within each individual microgel results in the physical hydrogel formation.²¹ Rheological study reveals the temperature dependence of gelation. The dynamic moduli of all microgels at 30 mg/mL are

plotted against temperature (Figure 2h–k). The stress was fixed at 0.1 Pa and the frequency at 0.1 Hz. At a lower temperature, the microgel dispersions are in the solid state. With an increase in temperature, both the elastic modulus (G') and loss modulus (G'') of the microgel dispersions increase sharply. The point at which the value of G' is equivalent to G'' is considered the gelation temperature T_{gel} . P(NIPAM-HEMA) has the lowest gelation temperature due to its hydrophobicity, a result of the highly hydrophobic comonomer, HEMA. Other microgels have a T_{gel} of around 32–33 °C. All the gelation temperatures are close to their VPTTs. This result implies that hydrophobic interaction is the main driving force for hydrogel formation. The hydrophilic microgels become hydrophobic when their temperature reaches their VPTTs, and the hydrophobic attractions drive the gelation of the microgels to form 3D structures. Furthermore, P(NIPAM-IA) has the highest mechanical strength, around 25 times higher than that of P(NIPAM-HEMA). Because the crosslinking within individual microgels is formed with additional intra- and inter-chain interactions, the large number of hydrophilic carboxyl groups from itaconic acid results in strong hydrogen bonding in comparison with the mostly hydrophobic HEMA.²¹

Characterization of hCSCs Three-Dimensionally Cultured with P(NIPAM)-Based Microgels

hCSCs used in the experiment were pre-screened by flow cytometry (Figure S2, Supporting Information). The CCK-8 assay was used to evaluate the metabolic activity of the cells, which reflects the viability and proliferation of hCSCs within the 3D microgel scaffolds. As shown in Figure 3a, at day 1, cells seeded in the microgels exhibit a similar absorbance when compared with two-dimensional (2D) culture, which indicates the high biocompatibility of the synthetic microgels as well as the same initial cell density for both 3D and 2D cultures. However, P(NIPAM-DMAEMA) halves their absorbance value in comparison with that of other microgels or 2D culture, which may be due to the positively charged DMAEMA microenvironment, since a positively charged microenvironment has a detrimental effect on cell viability.³⁸ Cell membranes are negatively charged and the positive microgel surface charge may rupture or damage them.

The cells in all microgels, except for the ones in the P(NIPAM-HEMA) microgel, are viable and grow in the first 5 days. The cell viability in the P(NIPAM-HEMA) microgel starts to drop after day 3. The small pore size of the P(NIPAM-HEMA) microgel has a high resistance to mass transport, which leads to nutrient and oxygen starvation, and the accumulation of toxic waste around the cells. Meanwhile, the highly hydrophobic surface of the P(NIPAM-HEMA) microgel prevents cell attachment and, therefore, leads to slow proliferation.³⁹ At day 7, the number of live cells in the positively charged P(NIPAM-DMAEMA) and the more hydrophobic P(NIPAM-HEMA) microgels are less than those in the other microgels. This indicates that the positively charged, highly hydrophobic microenvironments may not be appropriate for cell proliferation. In contrast, the cells in the P(NIPAM-IA) networks have the highest proliferation rate, which means that a hydrogel microenvironment with a slightly negative charge and a high degree of hydrophilicity may stimulate cell proliferation. A few other factors of the microenvironment may also contribute to better cell proliferation in this microgel: (i) the strong mechanical properties of the P(NIPAM-IA) microgel may provide the necessary physical support for easy cell attachment, thus, further promoting cell proliferation; (ii) the large average pore size of the

P(NIPAM-IA) network reduces its resistance to transporting oxygen, nutrients, and wastes to maintain high cellular viability; (iii) the extra carboxyl (COOH) group introduced into the microgel has been shown to improve cell attachment and proliferation studies in comparison with the ether group in PEGA and the hydroxyl (OH) group in HEMA;⁴⁰ (iv) the highly hydrophilic environment is beneficial for anchorage-dependent mammalian cell attachment and growth.⁴¹ In addition, hCSCs form spheroid structures which can upregulate cell adhesion molecules and proliferation mechanisms. Overall, hCSCs are more compatible with the microenvironment created by the P(NIPAM-IA) microgel, with its negative surface charge, strong hydrophilicity, large pore size, strong mechanical properties, and extra carboxyl groups.

To further investigate cell viability and morphology inside the microgels, LIVE/DEAD images were taken under a fluorescence microscope, as shown in Figures 3d–h and S3 (Supporting Information). hCSCs in the P(NIPAM-IA) microgel have the highest proliferation rate and the highest viability compared to those in the other microgels (Figure 3b,c). The morphology of hCSCs presents differently in the four microgels (Figure 3d–h). The morphology of hCSCs in the 3D microgel culture is round, which is similar to the cell morphology in the 3D cell culture.⁴² The P(NIPAM-DMAEMA) microgel has a positive surface charge, which helps cell attachment,³⁰ but restricts cell migration. P(NIPAM-HEMA) has the smallest pore size, which also restrains cell migration. Therefore, individual isolated hCSCs are spotted in both P(NIPAM-HEMA) and P(NIPAM-DMAEMA) microgel networks. On the other hand, hCSCs grown on the 2D culture plates display a flattened and spread-out shape. The formation of hCSC spheroids was reported to improve hCSCs viability and enhance their biological functions.^{18,43} Both the CCK-8 assay and the LIVE/DEAD images confirm that hCSCs are more viable and form larger clusters in microenvironments with more negative charges and a higher degree of hydrophilicity.

Biological Factors Released from hCSCs in Microgels

In an appropriate 2D culture microenvironment, hCSCs can release regenerative growth factors that play an important role in the survival and growth of cardiomyocytes.⁴⁴ Therefore, we examined the growth factors released from hCSCs in 3D culture. As shown in Figure 4a–c, hCSCs incubated in microgels release a higher amount of insulin-like growth factor-1 (IGF-1) at day 3 than those in the 2D culture, and all four microgels induce hCSCs to release IGF-1 to a similar degree. hCSCs release a similar amount of stromal-derived factor-1 α (SDF-1) in P(NIPAM-PEGA) and P(NIPAM-IA), as well as in 2D culture. At day 5, the amount of the vascular endothelial growth factor (VEGF) released from the cells in P(NIPAM-IA) is higher than those in the other microgels and in the 2D cell culture. At day 7, the VEGF is produced equivalently in P(NIPAM-PEGA), P(NIPAM-IA), and in the 2D cell culture. Overall, hCSCs show better performance within P(NIPAM-IA) and P(NIPAM-PEGA) microgels compared to the rest of the groups. The P(NIPAM-IA) microgel promotes multicellular spheroid formation due to its negative charge and porous structure. The gap junctions between cells in the hCSC spheroids can significantly promote cell proliferation and the release of growth factors.⁴³ In contrast, the microenvironments created in P(NIPAM-DMAEMA) and P(NIPAM-HEMA) gels have a negative impact on the release of SDF-1 and the VEGF from hCSCs. The VEGF released by hCSCs not only regulates the activation,

proliferation, and migration of vascular endothelial cells, but also augments angiogenesis.^{45,46} SDF-1 α guides stem cells to the injured heart tissue and also augments angiogenesis.⁴⁷ IGF-1 inhibits apoptotic cell death and enhances stem cell survival.⁴⁸ To further demonstrate the effect of these factors on cardiomyocytes, we collected conditional medium from hCSCs cultured in the microgels at day 5 and applied it to neonatal rat cardiomyocytes cultures (NRCMs) for 3 days. A LIVE/DEAD assay was employed to assess the viability of NRCMs. As shown in Figure 4d–h, the number of viable NRCMs cultured in the P(NIPAM-IA) microgel conditioned medium is the highest, while a similar number of viable NRCMs is obtained after culturing in the P(NIPAM-PEGA) microgel conditioned medium and in the 2D environment. The fewest NRCMs survive in the conditioned medium from the positively charged P(NIPAM-DMAEMA) and the hydrophobic P(NIPAM-HEMA) microgels. These results are in agreement with the those of the ELISA assays for growth factors VEGF and IGF-I, indicating that these two hCSCs factors contribute significantly to cardiomyocyte activation and survival. The microenvironment provided in the microgels is favorable for growing hCSCs and inducing them to produce the heart regenerative factors VEGF, SDF-1, and IGF-I.

In Vitro and in Vivo Evaluation of P(NIPAM-IA) Microgel Biocompatibility

To evaluate the biocompatibility of microgels in the heart tissue, we cultured neonatal rat cardiomyocytes (NRCMs) inside the microgels and on 2D culture plates as a control for up to 7 days. A LIVE/DEAD assay was employed to assess the viability and morphology of NRCMs inside the microgels (Figures 5 and S4 in Supporting Information). The results indicate that the highest number of viable NRCMs is found in the P(NIPAM-IA) microgel, which means that it may be the most biocompatible with NRCMs compared to the other microgels. Due to the varied surface charges of the microgels, NRCMs in these microenvironments present different morphologies. In a slightly positive or neutral environment, NRCMs interact with each other to form multicellular spheroids. In an environment with a high density of negative charges, they take on a round shape without exhibiting cell to cell interactions. NRCMs grown on the 2D culture plates show an elongated shape.

On the basis of the in vitro results, the P(NIPAM-IA) microgel was selected for further in vivo immunogenicity assessments in mice. Immune-competent male CD1 mice were intramyocardially injected with P(NIPAM-IA) microgel (Figure 6a). To evaluate the possible acute inflammation and T-cell immune reactions induced by the microgel, blood and hearts were collected 7 days after injections. Analysis of mouse inflammatory proteins shows that the plasma level of pro-inflammatory factors in mice treated with the microgel is comparable to that in control mice (Figure 6b), which indicates that injecting the microgel does not induce systematic inflammation. To evaluate the possible chronic inflammation induced by the microgel, hearts and spleens were collected 21 days after injections. The hematoxylin and eosin (H&E) staining revealed that the mice that were intramyocardially injected with the microgel did not present any discernable heart tissue inflammation (Figure 6c) compared to the positive control spleen sections (Figure 6d). Furthermore, the injection of microgels in the CD1 mice did not elicit obvious local T-cell immune rejection or exacerbate cardiac inflammation significantly. As shown in Figure 6e–g, the amount of CD3⁺ T cells, CD8⁺ T

cells, and CD68⁺ inflammatory cells (green) detected in the microgel injected hearts is similar to that found in the control hearts. The results suggest that the P(NIPAM-IA) microgel does not elicit obvious inflammation, local T-cell immune response, or macrophage infiltration.

Injectable Hydrogel-Encapsulated CSCs for MI Treatment

In our previous study, hCSCs embedded inside the hydrogel have demonstrated their therapeutic efficacy in treating MI.⁴⁹ To evaluate the therapeutic potential of P(NIPAM-IA) microgel-encapsulated hCSCs, we employed a mouse model of MI achieved by the ligation of the left anterior descending artery (LAD) (Figure 7a). Treatment with microgel-encapsulated hCSCs augments capillary densities in the post-MI heart after 3 weeks (Figure 7b). The expression of von Willebrand factor (vWF)-positive endothelial cells is much higher in the microgel-encapsulated hCSC-treated heart than in the heart injected with just microgel (Figure 7c). In addition to regenerating tissue, microgel-encapsulated hCSCs may also be responsible for the salutary preservation of tissues in the MI heart. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining reveals the anti-apoptosis effects of the microgel-encapsulated hCSCs. Less apoptotic nuclei are detected in hearts treated with microgel or microgel-encapsulated hCSCs compared to those in control hearts at day 7 (Figure 7d,e). These results indicate that the microgels used in this study may have innate antiapoptotic properties that are compounded by the addition of hCSCs.

Representative Masson's trichrome-stained myocardial sections show severe LV chamber dilation and infarct wall thinning in the nontreated hearts (Figure 7f) compared to microgel-treated hearts (Figure 7g) or microgel-encapsulated hCSC-treated hearts (Figure 7h) 3 weeks after treatment. Notably, microgel-encapsulated hCSC-treated hearts exhibit attenuated LV remodeling and less abnormal heart morphology with more viable myocardium (Figure 7i), smaller scar size (Figure 7j), and thicker walls (Figure 7k) after infarct. Left ventricular ejection fractions (LVEFs) were measured by echocardiography at baseline (4 h post infarct) and after 3 weeks. LVEFs are indistinguishable at baseline for all groups (Figure 7l). After 3 weeks, the LVEFs in MI controls (white bars, Figure 7h) are decreased while the microgel alone and microgel-encapsulated hCSC-treated animals exhibit LVEF preservation (blue bar, Figure 7m). When we calculate the change in LVEFs from baseline, MI controls show a drop in LVEF while microgel or microgel-CSC-treated hearts show an increase (Figure 7n). Attenuation in LV remodeling and preserved cardiac functions in the MI model indicate that the microgel stimulates endogenous repair mechanisms, in addition to providing mechanical support for the injured heart. This support includes an increase in wall thickness and a reduction in the wall stress of the damaged tissue.⁵⁰ Furthermore, the P(NIPAM-IA) microgel acts as a carrier for the transplanted cells, which resolves the problem of low retention rates for the therapeutic cell transplantation in the host. hCSCs contribute to the heart repair mainly through the paracrine effect,⁶ and the growth factor released from the cells may regulate augmented angiogenesis, inhibit apoptosis and promote endothelial cells' survival and activation. The introduction of the gel enhances stimulation of secretion of biological factors from the encapsulated stromal cells, and thus facilitates heart repair. In future studies, other P(NIPAM)-based formulations will be explored in vivo. In addition, the

biological mechanisms involved in the microgel-encapsulated CSC therapy need to be further elucidated.

CONCLUSIONS

We have examined the impact of P(NIPAM)-based hydrogel microenvironments, with varying degrees of surface charge and hydrophobicity, on hCSC growth and growth-factor release, as well as NRCM viability. The results demonstrate that hCSCs and NRCMs have higher viability and proliferate better when exposed to the negatively charged, hydrophilic microenvironment offered by the P(NIPAM-IA) microgel. hCSCs in the P(NIPAM-IA) microgel release regenerative growth factors which are vital for the growth and viability of NRCMs. The initial in vivo results show that injecting this microgel into mice does not elicit immune system responses or local T-cell/inflammatory cell infiltrations. The P(NIPAM-IA) microgels, combined with cardiac stromal cells, favors heart repair/regeneration in the MI mouse model. Our results demonstrate the therapeutic potential of P(NIPAM) thermo-responsive hydrogel scaffolds in cell-based cardiac tissue regeneration.

EXPERIMENTAL PROCEDURES

Microgel Synthesis

Emulsion polymerization was used to synthesize P(NIPAM)-based microgels according to our previous study.²¹ Detailed procedures can be found in Table 1. Monomers and surfactants were mixed with 97 mL of water in a 250 mL three-necked flask, equipped with a condenser and a mechanical stirrer. After 30 min of degassing, the flask was placed into a pre-heated oil bath at 70 °C. 3 mL of initiator aqueous solution (0.1 mmol) was injected to initiate polymerization. After 5 h, the reaction system was cooled down to room temperature and purified by membrane dialysis (cut-off M_w 12–14 kDa) against Milli-Q water for 1 week with daily water change. After purification, microgels were concentrated and stored in a fridge.

Derivation and Culture of Human CSCs

The methodology is taken from our previous publications using the cardiac explant method.^{51,52} Briefly, heart biopsies were harvested and minced into fragments of less than 2 mm³. Then, we digested the fragments with collagenase and seeded the tissue explants onto fibronectin-coated plates to create explant-derived cells (EDCs). In about 14 days, explant-derived cells were harvested and they can be maintained in the primary culture for several passages. Alternatively, the cells can be seeded in ultralow attachment flasks (Corning Life Sciences, Durham, NC) to form three-dimensional cardiospheres. Both EDCs and cardiospheres represent a natural mixture of intrinsic cardiac stromal cells from the heart.

Co-culture of hCSC and NRCM in Microgels

hCSC solution and 50 mg/mL of microgels (in PBS buffer) were mixed at a ratio of 2:3. 500 μ L of cell/gel mixture was seeded into each well of a 24 well-plate for a hCSC culture density of 10⁴ cells per well. hCSCs were further proliferated in 96 well plates with 50 μ L of cell/gel mixture in each well at a cell density of 2500 cells per well. After 1 h of incubation

at 37 °C to induce gelation, 150 μ L of cell culture medium was added to each well of a 96 well plate. The cells were incubated up to 7 days in a humidified environment with 5% CO₂. Culture medium was changed daily. After culturing for pre-determined days (1, 3, 5, 7 days), 10 μ L of cell count kit 8 (CCK-8) were added into each well to perform the cell proliferation assay for hCSCs. After 4 h of incubation at 37 °C, the gel/cell mixture was cooled down to room temperature until the gel liquefied. Then, the plate was placed into a microplate reader (Tecan Sunrise, Switzerland) to measure the absorbance at 490 nm.

NCRMs were derived as previously described.⁵³ NRCMs culture was performed in a 96 well plate with 50 μ L of cell/gel mixture in each well at a cell density of 1.5×10^5 cells per well. The NRCM culture and proliferation assays were conducted in the same manner as that for hCSCs.

ELISA and NRCM Cell Culture with Conditioned Medium

200 μ L of cardiac stromal cells (hCSCs) were mixed with 300 μ L of microgels at a density of 50 mg/mL (in PBS buffer, pH 7.2). The cell/ gel mixtures were seeded into 24 well-plates at a cell density of 10 000 cells/well. The plate was placed in an incubator at 37 °C for 45 min to allow for gelation of the microgel. 1 mL of cell culture medium was added into each well. The cells were cultured up to 7 days and received a medium change every 2 days. The cell culture medium was replaced by fetal bovine serum (FBS)-free medium and the cells were incubated for another 2 days. The FBS-free cell culture medium was collected for ELISA assays and for NRCM cell culture. The concentration of IGF-1, VEGF, and SDF-1 was determined by the ELISA kits following the manufacturer's instructions.

hCSCs were seeded in the microgel in a 48 well-plate. 400 μ L of DMEM was added into each well for 3 day culture and conditioning. 150 μ L collected conditioned medium was added into the each well of a 96 well-plate seeded with 7500 freshly harvested NRCMs. NRCMs were cultured with conditioned medium for 3 days. The LIVE/DEAD viability/cytotoxicity kit was used to assess cell viability.

Inflammation and Immunogenicity of P(NIPAM-IA) Microgels in Immunocompetent Mice

To assess the biocompatibility of P(NIPAM-IA) microgel in vivo, a cohort of male immunocompetent CD1 mice were anesthetized with isoflurane-oxygen inhalation. Their hearts were exposed through a minimally invasive left thoracotomy and randomized for 50 μ L P(NIPAM-IA) microgel injections. A group of mice was sacrificed 7 days post injections. Their blood was collected, and their hearts were harvested for immunochemistry. Another group was sacrificed at day 21. Their hearts and spleens were harvested for hematoxylin and eosin (H&E) staining. The hearts or spleens were frozen in optimum cutting temperature (OCT) compound and H&E staining was performed as described previously.^{54,55} Venous blood was harvested in an ethylenediaminetetraacetic acid (EDTA) tube and centrifuged for 20 min at 2000 rpm to obtain plasma, which was stored at -80 °C. A mouse inflammation antibody C1 array (RayBiotech, Norcross, GA) was used to quantify the inflammatory proteins in the plasma.

Mouse Model of Acute Myocardial Infarction

Male CD1 mice were anaesthetized with isoflurane-oxygen inhalation. Under sterile conditions, the heart was exposed through a minimally invasive left thoracotomy. MI was produced via the permanent ligation of the LAD coronary artery. Immediately after MI induction, the heart was randomized to receive one of the following three treatments: (1) “control (PBS)” group: intramyocardial injection of 50 μ L of PBS; (2) “microgel” group: intramyocardial injection of P(NIPAM-IA) microgels; (3) microgel + hCSC group: intramyocardial injection of microgel-encapsulated hCSCs. The MI mice treated with microgel-encapsulated hCSCs were intraperitoneally injected with rapamycin. Texas Red-X succinimidyl ester (Invitrogen, Carlsbad) at 1 mg/mL was used to pre-label hCSCs and/or microgels for detection.

Heart Morphometry

After the 3 week echocardiography study, animals were euthanized, and hearts were harvested and frozen in the OCT compound. Specimens were sectioned at a thickness of 10 μ m from the apex to the ligation level. 100 μ m intervals were used between each 10 μ m section. Masson's trichrome staining was performed according to the manufacturer's instructions (HT15 Trichrome Staining (Masson) kit; Sigma-Aldrich). The stained sections were imaged using a PathScan Enabler slide scanner (Advanced Imaging Concepts, Princeton). From these images, morphometric parameters were assessed using NIH ImageJ software, including the area of viable myocardium, scar size, and infarct thickness. Three sections were quantified for each animal.

Cardiac Function Assessment

The transthoracic echocardiography procedure was performed by a veterinary cardiologist who was blind to the experimental design. All animals were anesthetized in the supine position using an inhalable mixture of isoflurane and oxygen. Hearts were imaged in the long-axis views at the level of the longest LV diameter using a Philips CX30 ultrasound system, coupled with an L15 high-frequency probe. Ejection fractions were determined by measuring the images taken from the infarcted area. Baseline measurements were taken 4 h after injections and the end-point measurements were taken 3 weeks after injections.

Histology

For immunohistochemistry staining, frozen cryosections were fixed with 4% paraformaldehyde, permeabilized, and blocked with protein block solution (DAKO, Carpinteria, CA) containing 0.1% saponin (Sigma-Aldrich), and then incubated with antibodies overnight at 4 °C. The following are the primary antibodies used in this study: rabbit anti-CD3 (ab16669, Abcam, Cambridge, U.K.), mouse anti-CD8 α (mca48r, abd Serotec, Raleigh, NC), mouse anti-CD68 (ab955, Abcam) and rabbit anti-vWF (ab6994, Abcam). FITC- or Texas Red secondary antibodies (Abcam) were used in conjunction with the above primary antibodies. TUNEL solution (Roche Diagnostics GmbH, Mannheim, Germany) was applied to cryosections to evaluate their level of cell apoptosis. Images were taken with an Olympus epifluorescence microscope.

Statistical Analysis

All results were expressed as mean $\pm$ standard deviation (SD). Comparisons between two groups were conducted by two-tailed Student's *t*-test. One-way ANOVA with Bonferroni post hoc correction was used for comparisons among three or more groups. The difference between group means was considered statistically significant when the *p*-value was <0.05 .

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

X.C. would like to thank the University of Adelaide for providing scholarships. H.Z. would like to acknowledge the financial support from the ARC Discovery Project (DP160104632), University of Adelaide-NCSU Starter Grant, and the Medical Advances Without Animals (MAWA) Trust. J.T. and K.C. would like to thank the National Institute of Health (HL123920 and HL137093), the American Heart Association (18TPA34230092), the University of North Carolina General Assembly Research Opportunities Initiative grant, and the National Natural Science Foundation of China 81370216. English grammar was edited by Jhon Cores, PhD.

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Figure 1.

Schematic illustration of polymer synthesis and description of heart tissue regeneration using the human cardiac stromal cell (hCSC) spheroids cultured in thermosensitive injectable hydrogels. (a) Chemical structures of thermosensitive microgels. (b) hCSC spheroids cultured within the microgel network. (c) The conditioned culture medium collected from hCSCs spheroids within the microgel for neonatal rat cardiomyocytes (NRCMs). (d–f) hCSCs spheroid formation inside the microgel scaffold. (d) hCSCs are mixed with microgels at 25 °C. (e) At 37 °C, microgels confine single cells in a three-dimensional (3D) network. (f) After a pre-determined culture period, regenerative growth

factors are released from hCSC spheroids. (g) The growth factors released from hCSC can promote NRCM growth. (h) Injection of microgels into mouse hearts for immune response (without hCSCs) evaluation and MI treatment (both with or without hCSCs).

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Figure 2.

Characterization of microgels. (a) FTIR for P(NIPAM)-based microgels. The enlarged figures show the peaks for individual co-monomer functional groups; (b, c) temperature dependent microgel sizes: (b) hydrodynamic diameter (d_h) and (c) the shrinkage ratio $d_h(T)/d_h(25\text{ }^{\circ}\text{C})$ for 1.0 mg/mL P(NIPAM)-based microgels in physiological saline (pH $\approx$ 7.4); (d –g) SEM showing the morphologies of the in situ formed P(NIPAM)-based hydrogels (scale bar = 20 μm): (d) P(NIPAM-DMAEMA); (e) P(NIPAM-PEGA); (f) P(NIPAM-HEMA); (g) P(NIPAM-IA); (h–k) temperature dependence of the dynamic moduli of 30 mg/mL of P(NIPAM)-based microgels (pH $\approx$ 7.4): (h) P(NIPAM-DMAEMA); (i) P(NIPAM-PEGA); (j) P(NIPAM-HEMA); (k) P(NIPAM-IA). G' is elastic (or storage) modulus and G'' is viscous (or loss) modulus.

Figure 3.

In vitro analysis of hCSCs cultured in hydrogels and 2D culture. (a) Metabolic activity of hCSCs cultured in the 3D networks of P(NIPAM)-based microgels and 2D culture from day 1 to day 7. (b) Cell morphology analysis of hCSCs cultured in different microgels and 2D culture at day 7. (c) Viability of hCSCs cultured in different microgels and 2D culture at day 7 by LIVE/DEAD assay. (d–h) LIVE/DEAD images of hCSCs in P(NIPAM)-based microgels at day 7: (d) P(NIPAM-DMAEMA); (e) P(NIPAM-PEGA); (f) P(NIPAM-HEMA); (g) P(NIPAM-IA); and (h) 2D culture. Scale bar = 20 μm . Green is calcein (live cells) and red is EthD (dead cells). Data presented as mean $\pm$ standard deviation (SD) ($n = 3$). “*” indicates $p < 0.05$ for the comparison of cells cultured in P(NIPAM)-based microgels with those in 2D culture.

Figure 4.

Growth factors release from hCSCs cultured in different microgels. (a–c) Growth factors released from hCSCs in the P(NIPAM)-based microgel network and 2D culture at days 3, 5, and 7. (a) SDF-1; (b) VEGF; (c) IGF-1 (mean $\pm$ SD, $n = 3$). “*” indicates $p < 0.05$ for comparing cells in microgels with those in the 2D culture. (d–h) NRCM cell images in the conditioned medium. Conditioned medium was collected from different microgel networks at day 5. (d) P(NIPAM-DMAEMA); (e) P(NIPAM-PGA); (f) P(NIPAM-HEMA); (g) P(NIPAM-IA); (h) 2D culture. Green is calcein (live cells), red is EthD (dead cells), blue is Hoechst. Scale bar = 20 μm .

Figure 5.

NRCMs cultured in P(NIPAM)-based microgels. (a–e) LIVE/DEAD images of NRCMs in P(NIPAM)-based microgels at day 7; (a) P(NIPAM-DMAEMA); (b) P(NIPAM-PEGA); (c) P(NIPAM-HEMA); (d) P(NIPAM-IA); (e) 2D culture. (f) Cellular morphology analysis of NRCMs cultured in different microgels and 2D culture at day 7. (g) Cell viability of NRCMs cultured in different microgels and 2D plates at day 7 by LIVE/DEAD assay. Scale bar = 20 μm . Green is calcein (live cells) and red is EthD (dead cells). Mean $\pm$ SD ($n = 3$). “**”

indicates $p < 0.05$ for the comparison of cells cultured in P(NIPAM)-based microgels with those in 2D culture.

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Figure 6.

Biocompatibility test of P(NIPAM-IA) microgel in mice. (a) Schematic illustration of the overall animal study design. (b) Relative quantitative analysis of circulating levels of pro-inflammatory factors in mice injected with the P(NIPAM-IA) microgel (blue bar), or healthy control mice (white bar) at day 7 ($n = 3$ animals per group). (c) No obvious inflammation in the sections of hearts injected with the P(NIPAM-IA) microgel at day 21 by H&E stains. Red box presents the higher magnification of the injected area, and red arrow points toward the injected P(NIPAM-IA) microgel. (d) Normal spleen function in the mice injected with

the P(NIPAM-IA) microgel at day 21 by H&E stains. (e, f) Representative fluorescent images showing the presence of CD3⁺ T cells and CD8⁺ T cells (green) in the hearts injected with the P(NIPAM-IA) microgel, and normal control hearts at day 7 ($n = 3$ animals per group). (g) Representative fluorescent images showing the presence of CD68 + macrophage cells (green) in the hearts injected with the P(NIPAM-IA) microgel, or normal control hearts at day 7 ($n = 3$ animals per group). Scale bar is 200 μm . “*” indicates $p < 0.05$.

Figure 7.

P(NIPAM-IA)-encapsulated hCSCs to treat MI. (a) Schematic illustration of the design of animal experiments used to evaluate the therapeutic benefits of microgel-encapsulated cells in a mouse model of myocardial infarction (MI). (b) Representative images showing vWF-positive endothelial cells (green) in hearts treated by microgel alone or hCSCs in microgel ($n = 3$ hearts per group) at week 3. Scale bar is 100 μm . (c) The number of vWF-positive endothelial cells ($n = 3$ animals per group). (d) Representative fluorescent micrographs of TUNEL⁺ apoptotic cells (red) in hearts treated with microgel alone, or hCSCs in microgel,

compared to hearts with MI alone, at week 3. Scale bar = 100 μm . (e) The number of TUNEL⁺ apoptotic cells ($n = 3$ hearts per group). (f–h) Representative Masson’s trichrome-stained myocardial sections 3 weeks after treatment. (i–k) Quantitative analyzes of viable myocardium, scar size, and infarct thickness from the Masson’s trichrome images ($n = 5$ animals per group). (l–n) Left ventricular ejection fractions (LVEFs) measured by echocardiography at baseline (4 h post-MI) and after 3 weeks ($n = 6$ animals per group). “*” indicates $p < 0.05$ when compared to the “MI control” group; # indicates $p < 0.05$ when compared to the “MI + microgel” group.

Table 1.

Synthesis and Characterization of P(NIPAM)-Based Microgels

name	NIPAM (mmol)	co-monomer (mmol)	MBA (mmol)	SDS (mmol)	initiator (mmol)	ζ -potential (mV)			VPTT (°C)	T_{gd} (°C)
						25 °C	37 °C			
P(NIPAM-IA)	9.9	0.1 IA	0.2	0.2	0.1 KPS	-10.2 ± 0.2	-12.8 ± 0.5		34	32
P(NIPAM-PEGA)	9.9	0.1 PEGA	0.2	0.2	0.1 KPS	-1.9 ± 1.0	-1.8 ± 0.56		33	31
P(NIPAM-HEMA)	9.9	0.1 HEMA	0.2	0.2	0.1 KPS	-3.8 ± 1.1	-4.9 ± 0.4		30	27
P(NIPAM-DMAEMA)	9.9	0.1 DMAEMA	0.2	0	0.1 V-50	6.5 ± 0.5	7.6 ± 0.2		32	31