Heart Repair Using Nanogel-Encapsulated Human Cardiac Stem Cells in Mice and Pigs with Myocardial Infarction

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Supporting Information

ABSTRACT: Stem cell transplantation is currently implemented clinically but is limited by low retention and engraftment of transplanted cells and the adverse effects of inflammation and immunoreaction when allogeneic or xenogeneic cells are used. Here, we demonstrate the safety and efficacy of encapsulating human cardiac stem cells (hCSCs) in thermosensitive poly(N-isopropylacrylamide-co-acrylic acid) or P(NIPAM-AA) nanogel in mouse and pig models of myocardial infarction (MI). Unlike xenogeneic hCSCs injected in saline, injection of nanogel-encapsulated hCSCs does not elicit systemic inflammation or local T cell infiltrations in immunocompetent mice. In mice and pigs with acute MI, injection of encapsulated hCSCs preserves cardiac function and reduces scar sizes, whereas injection of hCSCs in saline has an adverse effect on heart healing. In conclusion, thermosensitive nanogels can be used as a stem cell carrier: the porous and convoluted inner structure allows nutrient, oxygen, and secretion diffusion but can prevent the stem cells from being attacked by immune cells.

KEYWORDS: cardiac stem cells, nanogel, myocardial infarction, biomaterials, mouse model, pig model

As a promising approach to tissue repair, multiple types of stem cells have entered the stage of clinical testing. However, their efficacy is limited by low retention and engraftment of transplanted cells, together with the potential risk of inflammation and immunoreaction when allogeneic or xenogeneic cells are used. Heart diseases including myocardial infarction (MI) and heart failure remain the leading cause of death worldwide. Even with the most advanced pharmacological and medical device treatment methods, mortality and morbidity of heart disease stay high. Cardiac tissue engineering and stem cell transplantation approaches aim at de novo cardiac regeneration after injury. Clinical outcomes of cardiac stem cell (CSC) therapy are hampered by low cell retention rate and side effects associated with immune rejection if allogeneic cells are used. Injectable hydrogels have been used to treat MI, and the studies have been demonstrated to improve cardiac function via LaPlace's Law (increased wall thickness and reduced wall stress). Various natural polymers such as fibrin, collagen, Matrigel, chitosan, keratin, and hyaluronic acid have been investigated as injectable hydrogels to treat MI. They have excellent biocompatibility and can promote cell migration, proliferation, and/or differentiation, leading to ultimate heart regeneration/repair. However, the drawbacks of natural polymers hampering their clinical applications are their batch-to-batch variation and expensive cost. Synthetic injectable hydrogels have been used to treat MI, and the studies have been demonstrated to improve cardiac function via LaPlace’s Law (increased wall thickness and reduced wall stress). Various natural polymers such as fibrin, collagen, Matrigel, chitosan, keratin, and hyaluronic acid have been investigated as injectable hydrogels to treat MI. 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Figure 1. Synthesis of P(NIPAM-AA) nanogel and characterization of nanogel-encapsulated CSCs. (A) Schematic showing the synthesis of P(NIPAM-AA) nanogel by emulsion polymerization. (B) FTIR spectra of P(NIPAM-AA) thermoresponsive nanogel. (C) Temperature-dependent hydrodynamic diameter, $d_h$, for 1 mg/mL of P(NIPAM-AA) nanogel in PBS. (D) Temperature-dependent shrinkage ratio $d_h(T)/d_h(25 \, ^{\circ}\text{C})$ in PBS. (E) Comparison of 30 mg/mL of P(NIPAM-AA) nanogel in PBS at sol state (25 °C) and gel state (37 °C). (F) Temperature-dependent dynamic rheological moduli of 30 mg/mL of P(NIPAM-AA) nanogel. Black closed circle corresponds to the elastic (or storage) modulus ($G'$), and the red circle corresponds to the viscous (or lose) modulus ($G''$). (G) Color-depth projection confocal image showing the morphology of CSCs encapsulated in the nanogel. Scale bar, 20 μm. (H) SEM image showing CSCs in the P(NIPAM-AA) nanogel. Scale bar, 20 μm. (I) Representative fluorescent image showing the morphology of CSCs cultured in nanogel. Scale bar, 10 μm. (J) Proliferation of CSCs cultured in P(NIPAM-AA) nanogel (red line) or on tissue culture plate (TCP) (blue line). (K–M) Release of insulin-like growth factor (IGF)-1, vascular endothelial growth factor (VEGF), and stromal cell-derived factor (SDF)-1 from hCSCs encapsulated in nanogel (red bar) or on TCP (blue bar) at various time points determined by ELISA. * indicates $P < 0.05$ when compared to the other group.
polymers hold the potential to replace natural polymers as injectable hydrogels to treat MI. For example, the copolymer of poly(N-isopropylacrylamide-co-acrylic acid) or P(NIPAM-AA) with hydroxyethyl methacrylate/poly(trimethylene carbo-

Figure 2. Impact of nanogel-encapsulated xenogeneic cardiac stem cells on cell retention and systemic inflammation in mice. (A) Schematic image indicating the general animal study design. (B) Ex vivo fluorescent imaging of mouse hearts at day 7 after injection of hCSCs in PBS or hCSCs in nanogel. (C) Quantitative PCR analysis of human cell retention in the mouse hearts (n = 3 animals per group). (D) Circulating levels of pro-inflammatory factors were remarkably elevated in mice treated with hCSCs in PBS compared to those treated with hCSCs encapsulated in polymer. (E–G) Fluorescent images revealing the presence of CD3+ T cells, CD8+ T cells, and CD68+ macrophage cells (green) in hearts injected with hCSCs (red) in PBS or nanogel at day 7 (n = 3 animals per group). Scale bar, 100 μm; * indicates P < 0.05.
(HEMAPTMC) has been used to treat chronic infarcted myocardium in animal models.27

One appealing regenerative medicine strategy for MI is encapsulating stem cells such as CSCs inside the hydrogels and deliver the cell-laden hydrogels into the damage tissues.28−30

Here, we propose the use of P(NIPAM-AA) nanogel, a synthetic injectable carrier to encapsulate human CSCs in mouse and pig models of MI. The nanogel serves as a scaffolding material to enhance cell retention and as a barrier to prevent T cells from entering and attacking the encapsulated CSCs. The treatment ultimately resulted in augmented cardiac function and stimulation of endogenous regeneration.

RESULTS

Synthesis and Characterization of P(NIPAM-AA) Nanogel. As an injectable hydrogel material, it is a solution at room temperature but changes into a soft gel at 37 °C. P(NIPAM-AA) nanogel was synthesized by emulsion polymerization (Figure 1A). Fourier transform infrared (FTIR) spectroscopy analysis was employed to identify functional groups of the synthesized nanogel (Figure 1B). Peaks at the bands around 1640 and 1550 cm⁻¹ as well as the peak of 1450 cm⁻¹ represent the chemical bonds of NIPAM.31,32 The band around 1710 cm⁻¹ is assigned to the C=O bond in AA, which is also confirmed by the titration (Supporting Information Figure S1A), indicating the successful copolymerization with NIPAM. Dynamic light scattering was applied to determine the hydrodynamic diameter (dh) of the nanogel dispersions at different temperatures in phosphate-buffered saline (PBS) to demonstrate that synthesized P(NIPAM-AA) is thermoresponsive (Figure 1C). The dh value dramatically decreases around a temperature of 30−35 °C, which corresponds to the volume phase transition temperature (VPTT) of this nanogel. To further illustrate the nanogel phase transition behaviors with temperature, the shrinkage ratio (dh(T)/dh (20 °C)) was plotted against temperature (Figure 1D). For a temperature beyond 30 °C, the shrinkage ratio decreases, signifying that the size of the nanogel shrinks. The nanogel sol−gel phase change was also observed (Figure 1E). At 37 °C, the balance between hydrophobic attractions and electrostatic repulsions offered by the carboxyl groups of the nanogel results in a gel state for the hydrogel.31 The rheological study was further conducted to characterize the mechanical properties of the nanogel shown in Figure 1F. At a lower temperature, the nanogel dispersions are at the sol state, where the loss modulus (G″) is greater than the elastic modulus (G′). The point at which the value of G′ is
greater than $G^\ast$ is considered as gelation temperature $T_{gel}$. From Figure 1F, the $T_{gel}$ of the nanogels is around 32–33 °C, which is close to their VPTT. Scanning election microscopy (SEM) was utilized to analyze the structure of the resultant hydrogel network (Supporting Information Figure S1B), which reveals the minuscule pore size of the network.

P(NIPAM-AA) Nanogel-Encapsulated Human Cardiac Stem Cells. Confocal microscopy (Figure 1G) and SEM (Figure 1H) revealed the morphology of human cardiac stem cells (hCSCs) in the P(NIPAM-AA) nanogel. Live/dead staining (Figure 1I; red = dead, green = live) showed 3D CSC clusters in the nanogel, distinct from the cell morphology on tissue culture plates (TCP, as control) (Supporting Information Figure S2A,B). Nevertheless, cell viability was not compromised by the nanogel (Supporting Information Figure S2C). Nanogel encapsulation did not affect the proliferation of CSCs (Figure 1J) and the release of various regenerative factors (Figure 1K–M), including insulin-like growth factor (IGF)-1, vascular endothelial growth factor (VEGF), and stromal cell-derived factor (SDF)-1 from CSCs.12

To test the biocompatibility of the nanogel with cardiomyocytes, neonatal rat cardiomyocytes (NCRMs) were cultured in the nanogel or on TCP for 7 days. Although NCRMs exhibited a unique morphology in the nanogel (Supporting Information Figure S3A–C) compared to their counterparts cultured on TCP, their viability was not compromised (Supporting Information Figure S3D). In addition, NCRMs exhibited similar cell viability when cultured in conditioned media collected from CSCs cultured on TCP or in nanogel (Supporting Information Figure S4A–C). The nanogel had no negative effects on NRCM contractility, which is an important cellular function of NRCMs (Supporting Information Figure S4D). These compound data sets indicated that the P(NIPAM-AA) nanogel was nontoxic to cardiac stem cells and cardiomyocytes.

Injection of Nanogel-Encapsulated CSCs Does Not Elicit Rejection in Normal Mice and in Pigs. To evaluate the systemic inflammation and local T cell infiltration to P(NIPAM-AA) nanogel-encapsulated hCSCs, immunocompetent CD1 mice were intramyocardially injected with hCSCs in PBS or hCSCs encapsulated in the nanogel. Mice were sacrificed 7 days after injection. Hearts and blood were collected for cell engraftment and systemic/local immune response analysis (Figure 2A). Ex vivo fluorescent imaging revealed that nanogel encapsulation significantly boosted cell retention (Figure 2B) in the heart. To have an accurate analysis of cell retention, quantitative PCR on human SRY gene was performed (Figure 2C). The results confirmed boosted cell retention from nanogel encapsulation. Mouse inflammatory protein array showed that the plasma levels of pro-inflammatory factors were remarkably elevated in mice treated with hCSCs in PBS compared to those treated with hCSCs.
encapsulated in nanogel (Figure 2D). This is consistent with previous studies indicating that xenogeneic CSC transplantation could induce systemic inflammatory response.\textsuperscript{13} Histology revealed greater numbers of Dil-labeled CSCs detected in heart (Figure 2E–G), confirming the \textit{ex vivo} imaging data. Micrographs of hematoxylin and eosin (H&E) staining also indicated no structure damage and T cell infiltration on spleen and heart sections obtained from mice injected with P(NIPAM-AA) nanogel at 21 days (Supporting Information Figure S5A,B). These data sets also confirmed that nanogel-encapsulated hCSC treatment did not elicit local T cell infiltration or exacerbate cardiac inflammation as only negligible amounts of CD3\textsuperscript{T} T 175 cells, CD8\textsuperscript{T} T cells, or CD68\textsuperscript{+} macrophage cells (green) were 176 identified in the heart (Figure 2E–G). Severe rejection was observed evidently in mouse hearts intramyocardially injected with human CSCs in PBS contrastively (Figure 2E–G). Additionally, injection of nanogel-encapsulated hCSCs in pig heart did not induce structural or functional damage of the kidney and the liver (Supporting Information Figure SSC–H).

**Nanogel-Encapsulated CSC Therapy Reduces Apoptosis but Promotes Angiomyogenesis.** Mouse model of MI was created by ligation of the left anterior descending artery (LAD) (Figure 3A). Immunocompetent normal CD1 mice were used. Immediately after MI induction, animals were randomized into the following four groups: (1) MI + hCSCs in nanogel, intramyocardially injected with \(1 \times 10^5\) hCSCs in 50 \(\mu\)L of P(NIPAM-AA) nanogel; (2) MI + hCSCs in PBS, intramyocardially injected with \(1 \times 10^5\) hCSCs in 50 \(\mu\)L of PBS; (3) MI + nanogel alone, intramyocardially injected with 50 \(\mu\)L of P(NIPAM-AA) nanogel; (4) MI control MI surgery without any treatment. Echocardiography was performed 4 h post-MI as the baseline and 3 weeks afterward as the end point. Less apoptotic nuclei were detected by TUNEL staining in hearts treated with nanogel-encapsulated hCSCs (red nuclei, Figure 3B,C). In addition, cycling cardiomyocytes (Ki67\textsuperscript{+/}alpha-SA\textsuperscript{+} cells; green nuclei, Figure 3D,E) were more evident in the hearts treated with nanogel-encapsulated hCSCs. Furthermore, treatment with nanogel-encapsulated hCSCs augmented capillary densities in the post-MI heart (Figure 3F–H). Also, patent blood vessels could be detected...
surrounding the injected nanogel-encapsulated hCSCs (Figure 3G).

**Nanogel-Encapsulated CSC Therapy Ameliorates Ventricular Dysfunction and Fibrosis in Mice with Acute MI.** Masson’s trichrome staining was performed 3 weeks after treatment (Figure 4A); the results showed that nanogel-alone treated heart (orange bars, Figure 4B–D) exhibited heart protection as compared to the MI (control) group (white bars, Figure 4B–D) to some extent. hCSCs injected in PBS did not confer any therapeutic benefits (blue bars, Figure 4B–D). Injection of hCSCs encapsulated in nanogel generated the largest therapeutic benefit in the MI heart (red bars, Figure 4B–D). Left ventricular ejection fractions (LVEFs) were detected at baseline (4 h post-infarct)
and 3 weeks post-MI. LVEFs from the four treatment groups were similar at baseline (Figure 4E). Three weeks later, the LVEFs in MI alone or hCSC-treated animals deteriorated continuously (white and blue bars, Figure 4F), whereas the nanogel-treated animals exhibited some degree of LVEF preservation (orange bar, Figure 4F). Injection of hCSCs in nanogel led to the highest LVEFs at 3 weeks (red bar, Figure 4F). When we calculated the treatment effects (i.e., change of LVEFs from baseline), it was clear that both MI alone and MI + hCSCs had negative treatment effects; nanogel alone preserved cardiac functions, and hCSCs in nanogel robustly boosted cardiac functions (Figure 4G).

**Nanogel-Encapsulated CSC Therapy in Pigs with Acute MI.** We then evaluated the therapeutic effects of nanogel-encapsulated hCSCs in a pig model of acute MI induced by LAD ligation (Figure 5A). Twenty minutes after ligation, mini-pigs were intramyocardially injected with PBS, hCSCs in PBS, or nanogel-encapsulated hCSCs. (Figure 5B). Injection of nanogel-encapsulated hCSCs did not increase the number of CD3-positive T cells in the post-MI heart (Figure 5C,D). Macroscopic images of pig heart indicated the infarct area on each heart slice (Figure 5E). Ex vivo fluorescent imaging revealed that nanogel encapsulation significantly boosted acute cell retention (Figure 5F) 24 h after injection in the pig heart. Four weeks after treatment, Masson’s trichrome staining revealed that nanogel-encapsulated CSC therapy reduced scar transmurality (Figure 6A). As an indicator of cardiac function, LVEFs were measured at baseline (before infarct), post-infarct (48 h post-infarct), and end point (4 weeks post-MI). LVEFs were similar at baseline for all groups and 4 h post-MI (Figure 6B). LVEF deterioration was evident in hearts treated with hCSCs but not in those treated with nanogel-encapsulated hCSCs over the 4 week time course (Figure 6C). Four weeks after treatment, cycling cardiomyocytes (alpha-SMA+ cells; green nuclei, Figure 6D) were more evident in the hearts treated with nanogel-encapsulated hCSCs. Furthermore, treatment with nanogel-encapsulated hCSCs increased the numbers of alpha-SMA+ vasculatures in the post-MI heart (Figure 6E).

**DISCUSSION**

Ischemic heart disease, especially MI, is the major reason for morbidity and mortality worldwide. Ischemia can cause irreversible loss of cardiomyocytes, followed by inflammation, fibrosis, and cardiac dysfunction. Despite the development of new medications and devices, heart failure can occur in a large number of MI patients. The therapeutic effects of stem cells in heart repair have been investigated in the last two decades. It has been clear that short-term cell retention rate and long-term cell engraftment rate were consistently poor in the heart regardless of the delivery routes and cell types. The poor vascularization of the injected area and the inflammation and immune reaction associated with allogeneic cell transplantation are the major hurdles for cell retention after delivery. We hypothesize that cell encapsulation technologies may overcome these hurdles.

Here, we synthesized thermosensitive poly(NIPAM-AA) nanogel with enhanced −COOH, which could provide a hydrophilic environment for cells proliferation and engraftment (Figure 1). It has been demonstrated that this material is able to promote stem cell proliferation and clustering, which leads to enhanced cell function and survival rate. The porous structure of the nanogel can maximize mass transport of nutrients, oxygen, and secretion of regenerative factors from the encapsulated cells (Figure 1 and Supporting Information Figure S1).

CSCs have been tested in laboratory animal model studies and in recent clinical trials for the treatment of MI. Like other cell types, CSCs also suffer from low retention after injection into the heart. In the present study, we investigated the potential of thermosensitive P(NIPAM-AA) nanogel-encapsulated human CSCs for the treatment of MI in both small (mouse) and large (pig) models.

Mounting lines of evidence have suggested that paracrine mechanisms play vital roles in CSC-mediated cardiac repair. CSCs secrete VEGF, IGF-1, and SDF-1, which can contribute to the neovascularization, inhibition of apoptosis, and recruitment of endogenous stem cells into the injured area. Nanogel encapsulation did not affect the viability and proliferation of CSCs and cardiomyocytes, suggesting its excellent biocompatibility (Figure 1 and Supporting Information Figures S2–S4). In addition, the release of various regenerative factors (including VEGF, IGF-1, and SDF-1) by CSCs was not affected by nanogel encapsulation (Figure 1).

One potential risk of allogeneic stem cell (including CSCs) transplantation is the possibility of triggering immune rejection and inflammation. Previous studies have demonstrated the benefit of using hydrogels to encapsulate and deliver stem cells to treat MI. However, in those approaches, either syngeneic models were used to avoid immune responses or the host immune system was suppressed to tolerate allogeneic or xenogeneic stem cells. Particularly, the test of human cells was normally done in immunodeficient animals. Here, we showed that injection of P(NIPAM-AA) nanogel-encapsulated hCSCs in immunocompetent mice did not trigger significant systemic inflammation or local infiltration of T cells and macrophages (Figure 2 and Supporting Information Figure SSA). In line with the absence of immune reaction, a larger amount of nanogel-encapsulated hCSCs was observed in the injected heart (Figure 2B,C,E–G), suggesting that nanogel encapsulation could enhance cell retention. In addition, injection of nanogel-encapsulated hCSCs in pig heart is nontoxic to the kidney and the liver (Supporting Information Figure S5C–H). These compound data sets suggested that the nanogel scaffolding material provided a barrier to prevent the entrance of T cells by the small pore size and capillary force generated by the porous structure.

The mechanisms underlying the therapeutic benefits of nanogel-encapsulated CSC therapy are likely to be complicated. Our findings indicated that the P(NIPAM-AA) nanogel-encapsulated hCSCs promoted post-MI cardiac repair by the inhibition of apoptosis and promotion of angiomyogenesis (Figure 3). Collectively, these favorable actions lead to reduced fibrosis and improved cardiac function (Figure 4). Fast degrading natural polymers do not support long-term support to the heart. In contrast, synthetic polymers cannot be quickly removed by enzyme activities.

Before one can start an IND-enabled human trial, normally large animal studies are needed to confirm the safety and efficacy end points observed in rodent models. We select the pig acute MI model because this model has been widely employed for stem-cell-based therapies for cardiac regeneration. In the pilot pig study, we confirmed that nanogel-encapsulated human CSCs did not elic it T cell infiltration but promoted cardiac function and angiomyogenesis in the post-MI heart (Figure S and Figure 6). Xenogeneic cells were used in our
study to exaggerate the immune reaction. In real scenarios, we expect the nanogel will provide a shield for allogeneic stem cells or induced pluripotent cells, which are likely to trigger immune reaction in the host tissue. In addition, the polymer carrier can drastically improve cell retention rate.

Our study has several limitations. First, we applied permanent vessel ligation in both mouse and pig models. Certainly, this is not what happens in real clinical situations where patients normally get coronary reperfusion. Second, minimally invasive delivery of biomaterials to the heart has been a challenge. In the current setting, open chest surgery is needed to expose the heart for direct muscle injection of the biomaterial/stem cell construct. Nevertheless, advanced equipment and technology have been developed to perform percutaneous endomyocardial injection (e.g., NOGA-Myostar injection).

CONCLUSION

Our findings indicated that synthetic porous nanogel can act as a favorable cell carrier for allogeneic/xenogeneic cell therapies. In particular, P(NIPAM-AA) nanogel blocks immune cells from entering while permitting the release of regenerative factors to promote regeneration. Taken together, nanogel-encapsulated hCSC therapy represents a safe and effective method for heart repair.

MATERIALS AND METHODS

Synthesis of Poly(P(NIPAM-AA)) Nanogel. N-Isopropylacrylamide (NIPAM, 99%), N,N-methylenediacrylamide (MBA, 98%), potassium persulfate (KPS, 99%), and sodium dodecyl sulfate (SDS, 98.5%) were bought from Sigma-Aldrich. Acrylic acid (AA, 99.5%) was purchased from Acros Organics Co. (New Jersey, USA). NIPAM was purified prior to synthesis through recrystallization in n-hexane and dried in vacuum at room temperature. Free radical emulsion polymerization was carried out to synthesize P(NIPAM-AA) nanogel. Based on the recipe in Table 1, 9.9 mmol (1.1203 g) of NIPAM, 0.1 mmol (6.86 mL) of AA, 0.2 mmol (31 mg) of MBA, and 0.2 mmol of SDS (57.9 mg) were dissolved in 97 mL of water. Then we poured the liquid into a 250 mL three-necked C oil bath, the system was degassed for 30 min. Three milliliters of KPS aqueous solution (0.1 mmol, 27 mg) was injected into the system to start the polymerization. The polymer synthesis was carried out for 5 h with continuous stirring under the protection of a nitrogen atmosphere at 70 °C. Once the polymerization was finished, the solution was purified by membrane dialysis (cutoff M, of 12–14 kDa) against Milli-Q water for a week with daily water change. After purification, nanogels were concentrated by heating to 70 °C. Two hundred microliters of the concentrated nanogel dispersion was dried at 70 °C for 48 h. The concentration was calculated.

Dynamic Light Scattering Measurement. A Zetasizer (Malvern, Nano-ZS) was utilized to measure the hydrodynamic diameter (d,) and ζ-potential of P(NIPAM-AA) nanogels (1.0 mg/mL in PBS buffer) at different temperatures. An autocorrelator was used to collect dynamic light scattering data.

Rheological Characterization. A universal stress rheometer SR5 (Rheometric Scientific) with a 40 mm cone plate geometry was used to perform dynamic oscillation experiments for 30 mg/mL nanogel dispersions. The gap was setup at 0.0483 mm, and the temperature was controlled by a Peltier system connected with a water bath. The elastic (storage) modulus G' and viscous (loss) modulus G" were examined at different temperatures from 20 to 40 °C. The stress was fixed at 0.1 Pa and the frequency at 0.1 Hz. The experiment was carried out in the linear viscoelastic region.

Hydrogel Morphologies. Nanogel dispersions (30 mg/mL) in physiological saline buffer (pH is approximately 7.2) were put into a 37 °C water bath to form physical gel. Once the gel was formed, the sample was quenched by liquid nitrogen and dried under vacuum using a Christ Alpha 2-4 LD free dryer. A Philips XL 30 FEG scanning electron microscopy was used to observe the hydrogel morphology after being coated with platinum at an acceleration voltage of 20 kV.

Derivation and Culture of Human Cardiac Stem Cells. Human CSCs were derived as previously described using the cardiogel method.24 In brief, heart tissues were cut into tiny pieces and washed with PBS and digestion of collagenase (Sigma, St. Louis, MO). Tissue fragments were cultured as “cardiac explants” on a plate coated with 0.5 mg/mL fibronectin (BD Biosciences, San Jose, CA) in IMDM supplemented with FBS, 0.5% gentamicin (Gibco, Life Technologies, California, USA), 0.1 mM 2-mercaptoethanol (In-vitrogen), and 1% l-glutamine (Invitrogen). After about 7–14 days, we collected cardiac explanted-derived cells with 0.25% trypsin (Gibco) and then seeded them in ultralow attachment flasks (Corning) for cardiospheres. After several days, cardiosphere-derived cardiac stem cells were formed by seeding harvested cardiospheres on fibronectin-coated plates and being incubated in 5% CO2 at 37 °C.

P(NIPAM-AA) Nanogel Encapsulation of Human CSCs. Human CSCs were collected in culture media and mixed with 10X PBS and 50 mg/mL P(NIPAM-AA) nanogel liquid in a ratio of 1:1:3 and then warmed in a 37 °C incubator for gelation to occur. The culture was maintained in IMDM (Invitrogen) containing 20% FBS. Human CSC morphology, viability, and proliferation in the nanogel were compared to that of hCSCs cultured on normal TCP. For cell viability, 1 x 106 hCSCs were cultured in 125 μL of P(NIPAM-AA) nanogel or on TCP on a 96-well plate for 7 days and then detected with the live/dead viability/cytotoxicity kit (Invitrogen). Cell morphology (e.g., cell body elongation) was calculated according to the image analysis results from ImageJ software. For cell proliferation, 1 x 105 hCSCs were seeded in 125 μL of P(NIPAM-AA) nanogel or on TCP on a 96-well plate, and we used counting kit-8 (Dojindo Molecular Technologies, Rockville, MD) to quantify cellular proliferation at days 1, 3, and 5. Absorbance rate was read by a microplate reader (Tecan Sunrise, Switzerland). Confocal images were captured by a ZEISS LSM 880 confocal microscope (Carl Zeiss, Oberkochen, Germany).

Scanning Electron Microscopy for P(NIPAM-AA) Nanogel-Encapsulated Human CSCs. The morphology of P(NIPAM-AA) nanogel-encapsulated hCSCs was studied by SEM (Philips XL30 scanning microscope, The Netherlands). The specimen was scanned and photographed under the microscope at an acceleration voltage of 15 kV.

In Vitro Cytokine Release of P(NIPAM-AA) Nanogel-Encapsulated Human CSCs. Three hundred microliters of 50 mg/mL of P(NIPAM-AA) nanogel (1x105 hCSCs) was placed into one well of a 24-well plate and incubated with 1 mL of FBS-free media. To study the continuous release of growth factors, we collected the conditioned media at day 3, 5, and 9 and added back fresh media into the well to be conditioned for the next time point. The expressions of IGF-1, VEGF, and SDF-1 in the conditioned media were determined by ELISA kits (R&D Systems, Minneapolis, MN; B-Bridge International, Cupertino, CA).
Biocompatibility of P(NIPAM-AA) Nanogel with Cardiomyocytes. To examine the biocompatibility of P(NIPAM-AA) nanogel, 1.5 × 10^5 neonatal rat cardiomyocytes (NRCMs) were collected in 25 μL of IMDM containing 10% FBS and mixed with 25 μL of 10x PBS and 50 mg/mL of P(NIPAM-AA) nanogel solution and then cultured on a 96-well plate in a 37 °C incubator for 3 days. NRCM morphology and viability in the polymer nanogel were characterized and compared to the NRCMs cultured on TCPS. The live/dead viability/cytotoxicity kit (Invitrogen) was applied to reflect cell viability and morphology. Beating NRCMs were observed using time-lapse imaging.

Secretion of CSCs Cultured in P(NIPAM-AA) Nanogel. Three hundred microliters of P(NIPAM-AA) nanogel (with 1×10⁵ hCSCs) was placed into each well of a 24-well plate and incubated with 1 mL of FBS-free media. As the control, 1×10⁵ hCSCs were seeded onto conventional TCPS. Conditioned media were collected at day 3 and plated onto NRCMs for 3 days. A live/dead viability/cytotoxicity kit (Invitrogen) was used for the determination of cell viability and morphology.

Immunogenicity of Polymer-Encapsulated hCSCs in Immunocompetent Mice. Male CD1 mice received one of the two treatments randomly: (1) hCSCs in PBS group, intramyocardial injection of 1×10⁵ human CSCs in 50 μL of PBS; (2) hCSCs in nanogel group, intramyocardial injection of 1×10⁵ human CSCs encapsulated in 50 μL of P(NIPAM-AA) nanogel. To enable fluorescent imaging and histological detection, hCSCs were labeled with red fluorescent Dil. Seven days after injection, mice were sacrificed to harvest the heart and blood. IVIS Xenogen In Vivo Imager (Caliper LifeSciences, Waltham, MA) was used for ex vivo fluorescent imaging. Afterward, the heart was frozen in OCT compound and sectioned at 10 μm thickness for histology analysis. Vein blood was harvested in a EDTA tube and centrifuged for 20 min at 2000 rpm to get plasma and stored in −80 °C. Mouse inflammatory antibody array C1 (Raybio, Norcross, GA) was used for the evaluations of inflammatory proteins in the plasma.

Mouse Model of Acute Myocardial Infarction. All animal work was approved by the Institutional Animal Care and Use Committee at North Carolina State University. Mouse MI model was generated as previously described.45,46 Generally, male CD1 mice were anesthetized by a minimally invasive left thoracotomy, and LAD was ligated to induce MI. After MI induction, the heart was to receive one of the following four treatments randomly: (1) MI + hCSCs in nanogel, intramyocardially injected with 1×10⁵ human hCSCs in 50 μL of P(NIPAM-AA) nanogel; (2) MI + hCSCs in PBS, intramyocardially injected with 1×10⁵ human hCSCs in 50 μL of PBS; (3) MI + nanogel, intramyocardially injected with 50 μL of P(NIPAM-AA) nanogel; (4) MI alone, MI surgery without any injection. The hCSCs or nanogels were prelabeled with Texas Red-X succinimidyl ester (1 mg/mL, Invitrogen) for detection.

Cell Engraftment Assay by Quantitative PCR. Animals were sacrificed, and their hearts were excised to obtain an actual measurement of the number of cells engrafted. Real-time PCR experiments using the human-specific repetitive Alu sequences were conducted. The whole heart was weighed and homogenized. Genomic DNA was isolated from aliquots of the homogenate with the DNAeasy minikit (Qiagen). The TaqMan assay (Applied Biosystems) was used to quantify the number of transplanted cells with the human Alu sequence as the template.

Hematoxylin and Eosin Staining. To evaluate possible immune responses to the injected nanogel, major organs from the injected mice and pigs were harvested. H&E staining was performed on tissue sections. Slides were fixed in hematoxylin (Sigma-Aldrich, MO, USA) for 5 min at room temperature and then rinsed in running water for 2 min. Afterward, the slides were decolorized in acid alcohol for 2 s and rinsed again in sodium bicarbonate for 5 dips, and the container was rinsed out with dehydrant after 95% iso for 30 s. Slides were then fixed in eosin solution (Sigma-Aldrich, MO, USA) for 2 min and then washed in 100% dehydrant (Richard-Allan Scientific, MI, USA) and subsequent xylene solution (VWR, PA, USA) three times. The slides were digitally photographed and analyzed by independent pathologists blinded to treatment allocations.

Heart Morphometry. After the echocardiography detection at 3 weeks, mice were euthanized and hearts were harvested and frozen in OCT compound. Specimens were sectioned at 10 μm thickness with 100 μm intervals. Masson’s trichrome staining was performed with a HT15 trichrome staining (Masson) kit (Sigma-Aldrich). Stained slides were placed in PathScan Enabler IV slide scanner (Advanced Imaging Concepts, Princeton, NJ) for image collection. NIH ImageJ software was used for the measurement of morphometric parameters in each section.47 Values from three sections per heart (5 hearts from each group) were determined and averaged.

Cardiac Function Evaluation. Under inhaled isoflurane–oxygen mixture anesthesia, the transthoracic echocardiography procedure was performed by a cardiologist and detected by a Philips CX30 ultrasound system coupled with a L1.5 high-frequency probe. Hearts were imaged 2D in long-axis views at the level of the greatest LV diameter. LVEFs were determined by measurement from views taken from the infarcted area.

Immunohistochemistry Staining. Heart cryosections were fixed with 4% paraformaldehyde, permeabilized, and blocked with protein block solution (DAKO, Carpinteria, CA) with 0.1% saponin (Sigma) and then incubated with the primary antibodies overnight at 4 °C. Primary antibodies were listed as follows: rabbit anti-c-D3 (ab16669, Abcam, Cambridge, United Kingdom), mouse anti-CD8 alpha (mc-48h, ab6306, Abcam), mouse anti-alpha-sarcomeric actin (a7811, Sigma), rabbit anti-Ki67 (ab15580, Abcam), rabbit anti-vWF (ab6994, Abcam), and a smooth muscle actin antibody (A5228, Sigma). FITC- or Texas-Red secondary antibodies obtained from Abcam Company were incubated and conjugated with related primary antibodies. For evaluation of cell apoptosis, heart cryosections were incubated with TUNEL solution (Roche Diagnostics GmbH, Mannheim, Germany) and counterstained with DAPI (Life Technology, NY, USA). Images were taken by an Olympus epi-fluorescence microscopy system as previously described.38,49

Pig Studies. Acute MI was induced in female mini-pigs (8–10 kg) by permanent ligation of LAD. Twenty minutes later, 10 million nanogel-encapsulated hCSCs were injected into the peri-infarct area in 10 sites (1 million for each site). Control animals received injection of hCSCs suspended in PBS. After the procedures, the animals recovered. Successful induction of MI was verified by ST elevation on an ECG. At three time points (baseline, 48 h post-MI, and 4 weeks after treatment), LVEFs were determined by echocardiography using a SIUI Apogee 1200v veterinary ultrasound system. Blood was collected at day 0 and day 28 for ALT, AST, urea, and creatinine analysis (DiaSys Diagnostic Systems). From the cryosections, Masson’s trichrome staining was performed, and images were taken from the infarct area. Scar transmurality was analyzed.

Statistical Analysis. All results are expressed as mean ± standard deviation. Comparison between two groups was performed by a two-tailed Student’s t test. One-way ANOVA test was used for comparison among three or more groups with Bonferroni post-hoc correction. Differences were considered statistically significant when P values were <0.05.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsnano.7b01008.

Additional biocompatibility of P(NIPAM-AA) nanogel, immune response to injected P(NIPAM-AA) nanogel in mice and pigs (PDF)

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J.T., J.Z., H.Z., and K.C. conceived the study; J.T., X.C.,
M.T.H., A.C.V., Y.H., D.S., and T.G.C. performed the
experiments and collected data; J.T., X.C., H.Z., and K.C.
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J.T., X.C., and T.G.C. contributed equally to this work.
Notes
The authors declare no competing financial interest.

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