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Long term culture of HL-1 cardiomyocytes in modular poly(ethylene glycol) microsphere-based scaffolds crosslinked in the phase separated state

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Abstract

Poly(ethylene glycol) (PEG) microspheres were assembled around HL-1 cardiomyocytes to produce highly porous modular scaffolds. In this study, we took advantage of the immiscibility of PEG and dextran to improve upon our previously described modular scaffold fabrication methods. Phase separating the PEG microspheres in dextran solutions caused them to deswell and crosslink together rapidly, eliminating the need for serum protein-based crosslinking. This also led to a dramatic increase in the stiffness of the scaffolds and greatly improved the handling characteristics. HL-1 cardiomyocytes were present during the microsphere crosslinking in the cytocompatible dextran solution, exhibiting high cell viability following scaffold formation. Over the course of 2 weeks, a 9-fold expansion in cell number was observed. The cardiac functional markers sarcomeric α -actinin and connexin 43 were expressed at 13 and 24 days after scaffold formation. HL-1 cells were spontaneously depolarizing 38 days after scaffold formation, which was visualized by confocal microscopy using a calcium-sensitive dye. Electrical stimulation resulted in synchronization of activation peaks throughout the scaffolds. These findings demonstrate that PEG microsphere scaffolds fabricated in the presence of dextran can support the long-term three-dimensional culture of cells, suggesting applications in cardiovascular tissue engineering.

Keywords

hydrogel; scaffold; HL-1 cardiomyocyte; phase separation; porosity

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Disclosure Statement

No competing financial interests exist.

1. Introduction

Advancements in cardiac tissue engineering (CTE) are currently limited by a number of engineering challenges. Engineered myocardium must contain a dense population of properly aligned and electrically connected cardiomyocytes. Scaffold materials must be extremely resistant to foreign body reaction because fibrous encapsulation might electrically isolate the engineered tissue and cause arrhythmias. Cardiomyocytes are highly metabolically active, form a well-coupled electromechanical syncytium, and must be in intimate contact with the vascular system [1, 2]. Scaffolds may also need to be highly porous to allow passage of nutrients so that cells survive even at the center of a large construct [3–5].

Most methods for producing porous scaffolds utilize processing conditions that are not compatible with cell survival. Consequently, cells must be seeded onto the exterior of a pre-formed scaffold. However, this may result in uneven cell distribution unless steps are taken to promote deeper cell penetration [6–9]. To achieve well distributed cell density, cytocompatible crosslinking and pore formation techniques may be needed. Bulk poly(ethylene glycol) (PEG) hydrogels are easily formed in the presence of cells with high cell viability [10–12]. However, the porosity of these scaffolds is at the nanoscale and methods to introduce pores are usually not cytocompatible [13–15].

A different approach is the formation of cell scaffolds by crosslinking hydrogel microparticles in the presence of cells. This technique introduces pores between the microparticles, which may provide greater nutrient diffusion and pathways for cells to migrate and proliferate. Sefton and colleagues have encapsulated cardiomyocytes and other cells in collagen-based modules, which were then coated with endothelial cells. The modules were stacked together, forming constructs with evenly distributed cells and endothelial cell-lined channels [16–18]. Additionally, Khademhosseini and colleagues have extensively engineered the self-assembly of cell-laden microgels [19–24]. Other modular approaches have been recently reviewed [25].

Modularity is desirable because different microparticles may perform different functions in the scaffold. We have recently developed scalable, aqueous methods for the fabrication of stable microspheres from eight arm PEG-amine (PEG₈-Am) and PEG₈-vinylsulfone (PEG₈-VS), as well as hydrolytically degradable microspheres from PEG₈-Am and PEG₈-acrylate [26, 27]. The microspheres were modified with arginine-glycine-aspartic acid (RGD) peptide to enable cell adhesion [28]. Microspheres had unreacted VS groups and aggregated by reaction with free amines in serum proteins, enabling assembly of microsphere-based scaffolds. By including both degradable and non-degradable microspheres, scaffolds with porosities on multiple length scales were fabricated *around* HepG2 liver cells. Some microspheres released sphingosine-1-phosphate (S1P), which promoted endothelial cell migration into the pores of the scaffold [27]. We have also utilized microsphere density differences to incorporate growth factor gradients into microsphere-based scaffolds [29].

We continued to engineer these scaffolds with the goal of developing functional, implantable cardiac tissues. Crosslinking of microspheres via serum proteins is undesirable because it may cause an antigenic response. Furthermore, the mechanical strength of scaffolds crosslinked by the previous method was limited. We hypothesized that by performing microsphere crosslinking in a cytocompatible dextran solution, phase separation between dextran in solution and PEG in the microspheres would result in microsphere aggregation and more rapid reaction kinetics. This may enhance the strength of the scaffolds and eliminate the need for serum proteins to crosslink the microspheres together. PEG/dextran aqueous two-phase systems have been widely used for cellular and molecular

partitioning [30–34]. Two-phase dextran/PEG solutions have also been used to produce microspheres and alter the porosity of hydrogels [35–39]. Dextran solutions were therefore seen as an attractive choice for enhancing the crosslinking of PEG microspheres in the presence of HL-1 cells (a cardiomyocyte cell line), allowing *in vitro* expansion and long-term culture in the resulting porous 3D scaffolds.

2. Materials and Methods

2.1. Synthesis of PEG derivatives and microsphere formation

Eight arm PEG-vinyl sulfone (PEG₈-VS) and eight arm PEG-amine (PEG₈-Am) were synthesized from eight arm PEG (Creative PEGworks) as previously described [40, 41]. Stock solutions of polymers (20%) in phosphate buffered saline (PBS) were sterilized by filtration (0.22 μ m, Millipore). For fluorescent labeling, 20% PEG₈-Am and Dylight-633 NHS ester (Pierce) were reacted overnight at 25°C at a 100:1 molar ratio of PEG:dye.

2.2. Microsphere formation

PEG₈-VS and PEG₈-Am stock solutions (20%) were mixed at a 2:1 volume ratio and pre-reacted at 37°C for 70% of the time needed to reach bulk gelation (5.25 h). The pre-reacted solutions were aliquoted and stored at –80°C. RGD cell-adhesion peptide (1 mM, sequence: GCGYGRGDSPG, Genscript) was added to the pre-reacted solution and reacted at 37°C for 30 min. For microsphere formation, PBS and 1.5 M Na₂SO₄ were pre-warmed to 37°C. Pre-reacted solution (50 μ L, room temperature) was then diluted with pre-warmed solutions in an Eppendorf tube to a final concentration of 2% PEG and 0.6 M Na₂SO₄ (500 μ L total). Phase separation occurred upon addition of Na₂SO₄. The mixture was triturated once and quickly transferred to a 37°C heating block. Reaction above the cloud point proceeded for 45 min. Microsphere suspensions were then diluted 3:1 in PBS, centrifuged at 14,100 g for 2 min, and the supernatant was replaced with an equivalent volume of PBS. This was repeated 2x to ensure all Na₂SO₄ was removed. After the final centrifugation, the pellet was resuspended in 500 μ L of PBS. The post-swelling microsphere volume was determined by measuring the total volume of solution after the addition of 500 μ L of PBS. Microspheres were stored in PBS at 4 °C and were stable for at least 6 months.

2.3. Microsphere sizing, swelling and aggregation

For microsphere sizing, aggregation was minimized by incubating microspheres in a 350 μ M cysteine solution for 2 hr at 25°C to cap free VS groups. Fluorescent microspheres (5 μ L) were suspended in 45 μ L of PBS, then 350 μ L of dextran in PBS was added just prior to imaging to attain the desired dextran concentration. Suspensions were vortexed for 30 sec, then samples (10 μ L) were imaged on a glass slide by confocal microscopy. Microsphere diameters ($n > 40$ microspheres per group) were measured using ImageJ software (National Institutes of Health) and volumes were calculated accordingly. Reported values are averages from 3 separately made microsphere batches, with error bars representing the 95% confidence interval.

To investigate dextran-induced aggregation, fluorescent microspheres were resuspended at a 1:1 volume ratio with 2%(v/v) fetal bovine serum (FBS, Sigma-Aldrich) or 20% or 40% dextran ($M_r = 100,000$, Sigma Aldrich) in PBS. Suspensions were vortexed and incubated at room temperature for 15 min. Aggregation of microspheres was assessed by fluorescence microscopy. Dextran concentrations are reported as %(w/v).

2.4. HL-1 culture

HL-1 cells, a gift from Dr. William Claycomb, are a continuously proliferating cardiomyocyte cell line derived from mouse atrial tumors [42, 43]. Cells were maintained

according to the protocol supplied by the Claycomb lab [44]. Briefly, Claycomb Medium (Sigma) was supplemented with 10% FBS, 100 µg/mL penicillin/streptomycin, 0.1 mM norepinephrine, and 2 mM L-glutamine. Supplementation with norepinephrine is key to maintenance of the contracting cardiac phenotype [42–44]. Cells from passages 50–70 were seeded at a density of $10^5/\text{cm}^2$ onto gelatin/fibronectin coated plates, supplied daily with 1 mL medium per 5 cm^2 culture area, and split 1:3 every 3.5 days. HL-1 monolayers were contractile by day 3 after passage.

2.5. Scaffold formation

Scaffolds crosslinked via serum proteins were fabricated as previously described [27]. For scaffolds crosslinked without serum proteins, dextran (20% unless otherwise noted) was dissolved in serum-free DMEM if cells were present or PBS otherwise. Scaffolds were made using 50 µL of RGD-containing microspheres. HL-1 cells were removed from flasks by trypsin dissociation, then rotated in 1.65 mL serum-free DMEM in an Eppendorf tube with the RGD-containing microspheres (10^7 HL-1s/mL microspheres) for 1 h at 37°C to promote cell adhesion (the “adhesion step”). The cells and microspheres were centrifuged at 500 g for 3 min and the supernatant was removed. Dextran-containing serum-free DMEM was added at a 1:1 volume ratio with the microspheres to resuspend the pellet. The suspension was centrifuged at 500 g for 3 min, then transferred to 37 °C/5% CO₂ for 1 h. Scaffolds were transferred to 6-well plates (BD Falcon) containing either Claycomb medium if cells were present, or PBS otherwise. Claycomb medium (4 mL) was replaced daily. Post-swelling dimensions were approximately 8 mm in diameter and 1 mm in thickness.

2.6. Microstructural and mechanical analyses of scaffolds

Confocal microscopy was used to enable measurement of scaffold porosity in the hydrated state. At least 8 confocal images per scaffold ($n=3$) were taken at randomly selected depths and analyzed in Matlab (MathWorks Inc.) to calculate percent porosity (area without microspheres/total area). Scaffolds for oscillatory shear rheometry (RFS3, Rheometric Scientific) were made using 100 µL microspheres and were crosslinked in Eppendorf tubes (PDMS was used to fill the conical portion of the tubes such that crosslinking occurred in the top of the tube). PBS-hydrated scaffolds (1 mm thick) were die cut to 8 mm and characterized using 8 mm parallel plates, with a normal force of 40 mN to prevent slipping. An amplitude sweep (G' as a function of strain) was performed to confirm that measurements were in the linear viscoelastic regime. Frequency sweeps (1–100 radians/second) were then performed at 5% strain.

2.7. Characterization of HL-1 response to scaffolds crosslinked in 20% dextran

For cell-viability analysis, scaffolds were incubated in serum-free DMEM containing calcein AM (2 µM) and ethidium bromide (4 µM) (Invitrogen) for 30 min. Ten images per scaffold ($n=3$) were taken at randomly selected depths and analyzed in Matlab to calculate percent viability. For measurement of scaffold cell densities, scaffold volume was measured prior to storage at -80 °C . At the time of the assay, scaffolds were thawed, mechanically dissociated by pipetting, and suspended to a final volume of 1 mL in dye/cell lysis buffer from a cell quantification kit (CyQUANT®, Invitrogen, excitation/emission: 480/520 nm). Suspensions were vortexed for 2 min, and 5 µL samples were diluted in an additional 195 µL of dye/lysis buffer on a 96-well plate. Spectrofluorometric measurements were compared to a standard curve of known HL-1 densities.

To ensure that cells were well distributed throughout the depth of the scaffold, a thicker (~1.65 mm), fluorescently labeled scaffold was fixed (24 hr after scaffold crosslinking) in 3.7% formaldehyde for 45 min at room temperature then treated with 30% sucrose solution for 3.5 h at room temperature for cryoprotection. Scaffolds were frozen in OCT (Tissue-

Tek®, Sakura) at -80°C for 30 min then cryostat sectioned with careful attention to depth of sectioning. Nine evenly spaced serial sections ($16\text{ }\mu\text{m}$ thickness) were treated with a nuclear stain (Hoescht 33258, Molecular Probes, 1:1000 in PBS) for 15 min at room temperature prior to imaging by fluorescence microscopy. The first 3 sections were designated as the top region of the scaffold, the middle three as the center, etc., with each region being $\sim 550\text{ }\mu\text{m}$ thick. Fifteen cell counts were made per region, averaged and divided by the average area of microspheres for that region.

For immunohistochemistry (IHC), non-fluorescent scaffolds were prepared for cryostat sectioning as described above. After sectioning, slides were blocked/permeabilized in 10% normal donkey serum/0.1% Triton X-100 in PBS. Primary antibodies were goat anti-connexin 43 (cx43, 1:100 dilution, Santa Cruz Biotechnologies, SC6560) and rabbit anti- α -actinin (1:500, Sigma Aldrich, A2543). Alexa Fluor 488-labeled donkey anti-goat (1:1000, Invitrogen, A11055) and Cy5-labeled donkey anti-rabbit (1:100, Chemicon, AP1825) secondary antibodies were used to visualize the staining. Coverslips were added with Vectashield® mounting medium containing DAPI (Vector Labs) for nuclear detection.

2.8. Microscopy

For scanning confocal microscopy (Eclipse C1/80i, Nikon), a hydrated scaffold was placed on a glass slide and covered with a circular glass coverslip ($150\text{ }\mu\text{m}$ thick). PBS was pipetted under the coverslip to prevent dehydration. For scanning electron microscopy (SEM, NanoSEM 2300, FEI Nova), hydrated scaffolds were fixed in 3.7% formaldehyde for 30 min at room temperature, suspended in dH_2O , frozen in liquid nitrogen, and freeze-dried overnight. Scaffolds were gold-coated (Cressington 108) and imaged at 15 kV. Images of fluorescent microspheres and immunohistochemistry (IHC) were acquired by fluorescence microscopy (IX70, Olympus) using a Magnafire camera (Optronics).

2.9. Detection of HL-1 electrical activity

Calcium sensitive Fluo-4 AM (10 mM in DMSO, excitation/emission: 494/516 nm, Invitrogen) was mixed (1:1 volume ratio) with 20% (w/v) Pluronic® F-127 (Invitrogen), diluted to $10\text{ }\mu\text{M}$ in DMEM, and applied to scaffolds for 30 min at $37^{\circ}\text{C}/5\%\text{ CO}_2$. Scaffolds were washed 3x in PBS and incubated 15 min in DMEM + 0.1 mM norepinephrine to allow for complete de-esterification of the Fluo-4. Scaffolds were rinsed once in Tyrode's solution (1.33 mM CaCl_2 , 1 mM MgCl_2 , 5.4 mM KCl, 135 mM NaCl, 0.33 mM NaH_2PO_4) containing 0.1 mM norepinephrine, 5 mM glucose, and 5 mM HEPES, and this solution was used to keep scaffolds hydrated during imaging. Norepinephrine is required in the culture media to maintain the beating phenotype over long culture periods, and was similarly added to the Tyrode's solution [42–44]. Electrical stimuli were delivered to tissue constructs via a bipolar electrode made of two silver microwires (8 mil, A-M systems Inc.) inserted under the glass cover slips and into the Tyrode's solution surrounding the scaffolds. Sequences of images (sampling rate = 1 – 2 Hz) were acquired by confocal microscopy at a depth of $100\text{ }\mu\text{m}$ into the scaffold while monophasic electrical impulses were applied to the scaffolds (amp = 10 V, duration = 200 msec, frequency = 1 Hz, burst width = 10 sec.) using an isolated pulse stimulator (Model 2100, A-M systems Inc.). Resulting images were analyzed using Matlab software for temporal patterns of fluorescence. Average fluorescence intensity was calculated over time for 8×8 pixel regions within the field of view, which were normalized to location-specific background intensity values to remove biases associated with the variability in cell density across the scaffold.

2.10. Statistics

Comparisons between 2 means were by Student's T-Test with $p < 0.05$ considered significant. Three or more means were compared by ANOVA with post hoc Scheffe test in

Matlab with $p < 0.05$ considered significant. Data are mean $\pm$ standard deviation or 95% confidence interval, as specified in the text.

3. Results

3.1. Effect of dextran on PEG microsphere swelling and aggregation

Individual microsphere diameters were used to calculate the average individual microsphere volume across a range of dextran concentrations. These were normalized to average volume of microspheres in PBS (diameter: $20.1 \pm 5.3 \mu\text{m}$, volume: $2109.8 \pm 1066.1 \mu\text{m}^3$). Microsphere volume did not significantly change in the presence of 2% serum (1.0 with a 95% CI of 0.8–1.3 as normalized to PBS alone), but decreased upon the addition of dextran, becoming more compact as dextran concentration increases (Fig. 1A). The wide variation in volume between microsphere batches at 5% dextran was because this was close to the critical concentration for phase separation. PEG concentration increases upon deswelling, enabling increased crosslinking between free VS and Am groups. Phase separation also causes microspheres in dextran solutions to aggregate, with increased packing at higher dextran concentrations (Fig. 1B; note that the dextran concentration prior to dilution 1:1 (v/v) with PEG microspheres is reported). This compaction should increase the local concentration of PEG for microsphere-to-microsphere crosslinking.

3.2. Fabrication and swelling of scaffolds crosslinked in 20% dextran

Due to the higher density of the dextran solutions, centrifugation resulted in microspheres rising to the top of the solution (Fig. 2A). Scaffolds made by our previous method (via serum proteins) required overnight immobilization for crosslinking into scaffolds that were strong enough to support their weight in solution, but could not be removed from the centrifugation container. Scaffolds fabricated in 20% dextran could be removed from their centrifugation containers after just one hour. The microsphere swelling data (Fig. 1A) helps to explain this phenomenon. Being a second order reaction, the 4-fold increase in PEG concentration in 20% dextran should increase the rate of the crosslinking reaction $4^2 = 16$ fold. Sixteen hours approximates the previously used overnight crosslinking step. Scaffolds that were crosslinked in dextran demonstrated substantial re-swelling upon transfer to physiological buffer (Fig. 2B).

3.3. Scaffold microstructure and mechanical properties

We hypothesized that the dextran-induced increases in local PEG concentration would give denser microsphere-to-microsphere crosslinking, leading to stronger scaffolds without the need for serum proteins. Parallel plate rheometry was used to quantify differences in stiffness. The storage moduli of scaffolds crosslinked in 20% dextran was approximately 3 times higher than the previously published scaffolds crosslinked in 2% serum (Fig. 3) [27]. As would be expected due to the increase in stiffness, scaffolds crosslinked in dextran better withstood routine cell culture procedures and assays.

Interestingly, confocal microscopy demonstrated larger pores in scaffolds crosslinked in dextran compared to scaffolds formed by centrifugation in 2% serum without dextran (Fig. 4). Scaffolds crosslinked in 2% serum without dextran were not highly porous, thus requiring porogens to enhance porosity [27]. These scaffolds also fractured easily, making it impossible to quantify their porosity by image analysis. Scaffolds crosslinked in the presence of 20% dextran were porous ($42.9 \pm 1.2\%$ porosity, $n=3$) even without addition of a porogen. We suspected that this was due to pre-crosslinking of the microspheres upon addition of dextran, perhaps around spherical dextran-rich droplets, leading to inefficient packing upon centrifugation. SEM images of these scaffolds are in general agreement with the scanning confocal images. However, it is clear that SEM drying artifacts make

interpretation of scaffold porosity much more tenuous, and that scanning confocal microscopy is clearly superior for characterizing porosity [45].

3.4. HL-1 incorporation, viability, and proliferation in microsphere scaffolds crosslinked in the presence of 20% dextran

Scaffold fabrication in dextran solutions was also possible in the presence of living cells, giving well distributed cell density throughout the scaffold's thickness (Supplementary Fig. 1). To enable cell adhesion to the microspheres, RGD peptides were reacted with PEG₈-VS during the pre-reaction stage, as previously described [27]. Cells in RGD-containing scaffolds had a more spread morphology, as expected (Fig. 5A). Cell viability 24 h after scaffold fabrication was $81.2 \pm 4.7\%$ (Fig. 5B, $n=3$). The cell density with 1 mM RGD-containing microspheres, measured 2 h after transfer of the scaffold to cell-specific medium, was 2.2 ± 0.3 million cells/mL scaffold ($n=3$), and only slightly higher by day 6 (Fig. 6A). However, by day 13, HL-1 cells had proliferated to a density of $18.3 \pm 1.0 \times 10^6$ cells/mL scaffold (Fig. 6A, $n=3$), and much of the porous space in the center of the scaffold was filled with HL-1 cells (Fig. 6B). Proliferation of cells resulted in the formation of large cell clusters in the pores of the scaffold that changed the macroscopic appearance of the scaffold (Supplementary Fig. 2).

The experimental design of the proliferation assay originally contained control scaffolds that were crosslinked via serum proteins. However, they broke if removed from their centrifugation containers. In our previous study [27], scaffolds were maintained in the centrifugation containers and the latest time point was 108 hr (meaning 2–3 media changes). It is difficult to maintain serum protein-based scaffolds in centrifugation containers much longer than that because frequent media changes cause scaffold breakage.

3.5. HL-1 expression of cardiac functional markers in microsphere scaffolds crosslinked in the presence of 20% dextran

When cultured on two-dimensional substrates, HL-1 cells are known to express both connexin 43 and sarcomeric α -actinin, which are key proteins in excitation and contraction of myocytes, respectively [42, 43]. Punctuated expression of the gap junction connexin 43 indicates connexon/gap junction assembly and suggests intercellular electrical connectivity. These markers were probed by immunohistochemistry at days 13 (Fig. 7A–C) and 24 (Fig. 7D–F) after crosslinking. Extensive proliferation (Fig. 7E–F) and cell clustering (Fig. 7D) was further confirmed by nuclear staining at day 24. Both connexin 43 (Fig. 7B and 7E) and sarcomeric α -actinin (Fig. 7C and 7F) were expressed at day 13 and continued to be expressed through day 24 (Fig. 7). The continued expression of these proteins suggested that the cells were electrically connected and retained their cardiomyocyte phenotype during culture in the microsphere scaffold.

3.6. Detection of HL-1 electrical excitation in microsphere scaffolds crosslinked in the presence of 20% dextran

As a final test of maintenance of cardiac phenotype, we looked at HL-1 excitation after over 5 weeks in culture. Activation of HL-1 cells in day 38 scaffolds was detected by confocal microscopy of Fluo-4 stained cells (Fig. 8; movies available as supplementary materials). Fluorescence intensity traces were collected for multiple pixels in different regions of interest (ROI). Pixel traces shown in a single ROI graph are spaced by a distance of at least 16 μm , such that some of the traces were likely from adjacent cells. Spontaneous excitation was observed throughout the scaffolds (Fig. 8A), with pixels from various areas of a single ROI peaking simultaneously. This suggests some degree of electrical connectivity between cells of a single cluster. The duration of the spontaneous transients varied between ROIs, possibly due to variation in the activity of Ca^{2+} -ATPase on the surface of the sarcoplasmic

reticulum. In paced scaffolds, cells in various ROIs were excited at the same frequency (0.5 Hz) for the 10 s duration of the stimulus (Fig. 8B). The excitation frequency of these clusters was only half the stimulation frequency (i.e. a 2:1 block), suggesting that the cells have a long refractory period.

Contraction of cell clusters was not visualized at the microscopic level (see supplementary movies), but the use of an excitation-contraction uncoupler (e.g. blebbistatin) would be necessary to absolutely verify that transients were not influenced by contraction-induced motion. Although the traces do not appear to represent pure action potential, the presence of calcium transients and/or motion indicate that the cells are still electrically active after 5 weeks in the 3D scaffolds. This suggests a promising outlook for future work investigating HL-1s or other types of cardiomyocytes. Future studies will focus on optimization of materials to support a contractile phenotype.

4. Discussion

Phase separations have been utilized to produce porous hydrogel materials for at least five decades [46–48]. Crosslinking of a polymer above its LCST results in the formation of sponge-like morphologies that sometimes appear to be aggregates of microspheres [45, 49–51]. The current method differs from traditional phase separation methods because scaffold formation is divided into a microsphere formation step and a microsphere assembly step. Like our previously reported methods [26, 27], the microsphere formation step is performed in sodium sulfate solutions rather than dextran because the microsphere yield is much higher. The scaffold formation step is then performed in cytocompatible dextran solution, which provides a stiffness increase that enables more extensive biological characterization than was possible using the serum protein-based protocol. The two-step method allows the fabrication of large quantities of high quality microspheres that may be stored for longer than six months prior to scaffold formation. It is our experience that the complexity of processing materials in the phase-separated state is mostly captured in the first step. Thus, if the first step yields high quality microspheres, the same microsphere batch may be used over the course of many experiments.

Materials used for cardiac tissue engineering are typically: 1) macroporous scaffolds formed apart from cells, or, 2) non-macroporous hydrogels crosslinked around cells. As an example of pre-formed scaffolds, Langer and colleagues produced highly porous poly(glycerol sebacate) materials that had mechanical properties similar to myocardium [52]. The scaffolds were engineered with intricate, biomimetic pore structures that promoted the alignment of cardiomyocytes [53]. As an example of a non-macroporous hydrogel, collagen gels have been extensively characterized by Eschenhagen, Zimmermann, Elson and colleagues [5, 54–59]. Primary heart cells were added to collagen solutions prior to gelation. Cardiac fibroblasts contracted the gels, which resulted in highly aligned, electrically active tissues [5, 54–59]. While the lack of porosity may place size limitations on the tissues, researchers have circumvented this problem by fusing multiple small collagen gels to create larger cardiac patches [57–59].

Materials for cardiac tissue engineering materials must be especially resistant to the formation of a fibrous capsule, which may result in electrical isolation of the transplanted tissue. Because of its low protein adsorption properties, PEG is an excellent candidate for these types of applications [60–62]. The PEG-based scaffolds described here are macroporous when assembled in the presence of living cells. Similar to traditional macroporous scaffolds, the cells have space within the pores to migrate and proliferate. Similar to a collagen gel, the cells are evenly distributed regardless of scaffold thickness

from the time of scaffold formation. The storage modulus is also higher than reported values for collagen gels (10–100 Pa) [63–66].

Collagen gels are typically remodeled by cardiac fibroblasts that are present in the primary heart cell harvests. Unfortunately, proliferating fibroblasts may quickly overtake the cardiomyocytes, limiting the window of study to a few days and making it difficult to analyze cardiomyocyte-specific responses [57, 67]. While patient-derived stem cells may be the optimal cell source for cardiac tissue engineering, HL-1 cells are a useful model for studying cell responses to biomaterials because they maintain many of the characteristics of cardiomyocytes. HL-1 monolayer cultures have previously been used to probe cardiomyocyte responses to various drugs and other treatments [43, 68–70].

Several recent studies have used HL-1 cells to study cardiomyocyte responses to biomaterials [71–74]. For example, Stupp and colleagues demonstrated HL-1 alignment and electrical activity at day 10 in nanofibrous amphiphilic peptide gels [71], but viability and retention of cardiac phenotype in longer-term cultures were not investigated. Jongpaiboonkit *et al.* investigated viability over time for HL-1s encapsulated in bulk PEG-diacrylate hydrogels, which have negligible porosity [74]. Viability in non-degrading bulk gels at day 1 was slightly higher than what was seen in the PEG microsphere scaffolds (~90% versus ~81%, respectively). This slight viability difference may be a product of the length of the adhesion step used to ensure HL-1 adhesion to microspheres during centrifugation. At two weeks, HL-1 viability in the bulk PEG hydrogels was low unless RGD density and degradation rate were finely tuned [74]. In the current study, HL-1 cells expressed functional markers and had a nine-fold increase in cell density at two weeks. While a long-term comparison between the systems has not yet been performed, HL-1 cells in PEG microsphere-based scaffolds may be at a long-term advantage due to increased porosity and nutrient diffusion.

However, the current scaffolds still need to be improved to be useful for cardiac tissue engineering. While dense cardiomyocyte packing is important for conduction, localized HL-1 proliferation caused clustering within the pores of the scaffold, and the cells lacked the alignment and highly organized gap junctions (Figs. 6 and 7) typical of natural myocardium. Furthermore, the frequency of spontaneous transients in the scaffolds was lower than those reported for *confluent* HL-1 monolayers [75] and seen in our own two-dimensional cultures, but similar to what is reported for *non-confluent* monolayer cultures [68]. The lower frequency and lack of synchronization in spontaneous transients probably indicates low electrical conduction between cell clusters in the scaffold. Functionalizing some of the PEG microspheres with enzymatically degradable sites may enhance porosity and help the cells to remodel their environment, similar to what is seen in collagen gels [55, 76]. Coupling enzymatic degradation with mechanical [56] and/or electrical [77] stimulation may further improve cellular alignment and electrical conduction.

Finally, replacing the HL-1 cells with an autologously derived cardiomyocyte source will be necessary before the engineered tissue can be used in a clinical setting. Recent studies have found that matrix stiffness [78–80] and topography [81–83] have profound influence on cell differentiation and maturation, so it is possible that the mechanical differences observed in this study would affect cell phenotype. However, the microstructural differences between PEG microsphere scaffolds and those described by other groups limit our ability to hypothesize on the outcome of mechanical and topographical changes. Our future research will likely include variation of microsphere size and stiffness and its effects on stem cell differentiation and cardiomyocyte phenotype.

5. Conclusions

In this study, previously published modular scaffold fabrication methods were improved, developing stronger, more porous PEG scaffolds by crosslinking microspheres in the phase separated state. Scaffolds were assembled around HL-1 cardiomyocytes, and cells were cultured in the scaffolds for over a month. The HL-1 cells proliferated, expressed cardiomyocyte markers, and retained electrical activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Phase separation in dextran increases localized PEG concentration through microsphere deswelling and aggregation

(A) The effect of dextran concentration on microsphere size was studied. Fluorescent microspheres were suspended in dextran solutions of varying concentrations. Care was taken to diminish microsphere aggregation prior to confocal imaging. Microsphere volumes (■) were determined by image analysis ($n=3$ microsphere batches, >40 microspheres per batch) and plotted alongside corresponding microsphere PEG concentrations (▲) (both normalized to PBS alone =1). Microspheres showed a decreasing size trend with increasing dextran concentrations. Error bars correspond to the 95% confidence intervals at each point. (B) Pelleted fluorescent microspheres were resuspended at a 1:1 volume ratio in 2% fetal bovine serum, 20% dextran or 40% dextran, vortexed for 1 minute, allowed to aggregate at 25°C for 15 min, and imaged by fluorescence microscopy. Magnified insets highlight the packing of multiple microspheres to form large aggregates at higher dextran concentrations.

Figure 2. Microsphere scaffold formation and swelling

(A) Microspheres were suspended in medium containing either 2% serum or 20% dextran, centrifuged, then crosslinked to form scaffolds. Phase separation in dextran allowed microsphere crosslinking in the absence of serum proteins. Scaffolds formed in dextran were much stronger and could be transferred to cell culture plates and cell-specific medium after only 1 h at 37°C. Scaffolds crosslinked in 2% serum had to be kept in the same tubes in which they were formed to avoid breakage. (B) To demonstrate microstructural changes caused by swelling upon dextran removal (after crosslinking step), a scaffold crosslinked in 40% dextran was imaged by confocal microscopy before and after buffer exchange into PBS.

Figure 3. Scaffolds crosslinked in the presence of 20% dextran are stiffer

Storage moduli (G') of microsphere scaffolds crosslinked in either 20% dextran (n=3) or 2% serum (n=2) in PBS, 1 d after scaffold formation. Error bars = std. dev. *p < 0.05 for all frequencies

Figure 4. Scaffolds crosslinked in the presence of 20% dextran have increased porosity
Hydrated scaffolds containing fluorescently labeled PEG₈-Am were imaged by scanning confocal microscopy (top row). Fixed and dehydrated scaffolds were also analyzed by scanning electron microscopy (bottom row). Microsphere-based scaffolds were compared to bulk PEG hydrogels made from the same precursors by Michael-type reaction (2:1 PEG₈-VS:PEG₈-Am). Scaffolds formed in dextran were porous without the addition of porogens. Scaffolds formed in 2% serum appeared to be porous, but these are actually large fractures, and the microspheres are otherwise tightly packed.

Figure 5. HL-1 cells retain high cell viability in scaffolds assembled in the presence of 20% dextran

(A) Scaffolds (blue) were formed from microspheres with and without RGD peptide. HL-1 cells were mixed with the microspheres and rotated for 1 h at 37°C to promote attachment of the cells to the microspheres prior to scaffold formation in 20% dextran. One day after scaffold formation, HL-1 cells were labeled with calcein AM (green) and imaged by scanning confocal microscopy. Cells were more spread in scaffolds with RGD peptide. (B) Live/dead assay of HL-1 cells in a scaffold 1 d after cross-linking, as imaged by confocal microscopy. Cell viability was $81.2 \pm 4.7\%$ ($n = 3$).

Figure 6. HL-1 cell proliferation in scaffolds

(A) HL-1 cell density immediately following scaffold formation (day 0) and after 6 and 13 days. Cell density increased nine-fold by day 13. * $p < 0.05$ versus day 0 and day 6. (B) HL-1 cells labeled at day 0 with Cell-Tracker dye (green) then incorporated into scaffolds (blue) to follow the increase in cell density over time. Scanning confocal images are from the interior of each scaffold.

Figure 7. HL-1 cells express cardiac functional markers after long term culture in microsphere-based scaffolds crosslinked in 20% dextran
 Immunohistochemical staining for connexin 43 (green), sarcomeric α -actinin (red), and DAPI (blue) at: (A–C) day 13 and (D–F) day 24 after scaffold formation. Magnified staining for (B and E) connexin 43/DAPI alone and (C and F) sarcomeric α -actinin/DAPI alone. Connexin 43 staining was punctuated suggesting substantial cell-cell contacts, while sarcomeric α -actinin expression indicated that cells had retained the cardiomyocyte phenotype. Section slices were 10 μ m thick for all images. Images were acquired by fluorescence microscopy.

Figure 8. HL-1 cells retain electrical activity after 38 days of culture within microsphere-based scaffolds crosslinked in 20% dextran

Day 38 scaffolds containing HL-1 cells were incubated with calcium-sensitive Fluo-4 AM to monitor changes in intracellular calcium concentrations. Activity was imaged by scanning confocal microscopy at a depth of 100 μm into the scaffolds. (A) Spontaneous transients were seen in various regions of interest (ROI) throughout the scaffold. (B) Transients from various ROI in response to stimulation by monophasic electrical impulses (amplitude = 10 V, duration = 200 msec, frequency = 1 Hz, burst width = 10 sec). Traces of transients from different ROI or random pixels are shown. The multiple traces from within each ROI were from pixels spaced at least 16 microns apart.