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Clickable Poly(ethylene glycol)-Microsphere-Based Cell Scaffolds

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Abstract

Clickable poly(ethylene glycol) (PEG) derivatives are used with two sequential aqueous two-phase systems to produce microsphere-based scaffolds for cell encapsulation. In the first step, sodium sulfate causes phase separation of the clickable PEG precursors and is followed by rapid gelation to form microspheres in the absence of organic solvent or surfactant. The microspheres are washed and then deswollen in dextran solutions in the presence of cells, producing tightly packed scaffolds that can be easily handled while also maintaining porosity. Endothelial cells included during microsphere scaffold formation show high viability. The clickable PEG-microsphere-based cell scaffolds open up new avenues for manipulating scaffold architecture as compared with simple bulk hydrogels.

Keywords

biomaterials; click chemistry; hydrogels; microsphere scaffolds; poly(ethylene glycol)

1. Introduction

Polymeric materials are promising as scaffolds for tissue reconstruction and drug delivery systems for the therapeutic release of bioactive molecules.^[1–3] Poly(ethylene glycol) (PEG) is a widely used hydrophilic polymer that resists non-specific biological interactions,^[4–6] yet is readily modified with proteins and peptides for enzymatic biodegradation and cell adhesion.^[7–9] While PEG hydrogels have been demonstrated to be useful in a number of applications, modular or self-assembling hydrogel-based scaffolds promise to facilitate the generation of complex architectures that better mimic the organization of natural tissues.^[7,10] PEG scaffolds and hydrogels have been crosslinked using a wide variety of chemical reactions including free-radical polymerization,^[11] Michael-type addition,^[12] enzymatic reaction,^[13] irradiation of linear or branched PEG polymers,^[14] mixed-mode polymerizations,^[15,16] native chemical ligation,^[17] and other strategies.^[6,9] Click reactions

are defined as bioorthogonal reactions and include reactions such as the Huisgen 1,3-dipolar cycloaddition between azides and alkynes, thiol-ene/yne photoadditions, and Staudinger ligation.^[18–22] Taking advantage of the rapid reaction kinetics of copper(I)-catalyzed azide–alkyne cycloaddition, crosslinked hydrogel networks have been produced and tailored for various applications.^[23–30] Crosslinked polymer networks have also been fabricated and modified for several applications using thiol-ene/yne photoadditions.^[29,31,32] Copper-free azide–alkyne cycloadditions have recently attracted attention for materials fabrication, as these reactions have high conversions, fast kinetics, insensitivity to oxygen and water, stereospecificity, regiospecificity, and mild reaction conditions.^[33–36] Additionally, the reactions can be performed under physiological conditions with little risk of non-specific reactions with molecules found in cells and tissues.^[20] Copper-free cycloadditions have been designed to allow incorporation of protease-sensitive peptides to enable enzymatic biodegradation^[30,34,35,37] and attachment of cell adhesion peptides through photoaddition.^[29,34,35,37] Due to the exceedingly slow reaction kinetics of alkynes with thiols, amines, alcohols, etc., PEG scaffolds formed using click reactions may be useful for cell transplantation and drug delivery.

We previously developed techniques for the fabrication of PEG microspheres by phase inversion polymerization.^[38–40] By lowering the lower critical solution temperature (LCST) of PEG with kosmotropic salts, reactive PEG derivatives underwent a thermally induced phase separation. Spherical PEG-rich domains coalesced and rapidly increased in size due to the absence of surfactants or stirring. However, coalescence was halted when the spherical domains reached the gel point.^[39] We previously found that the mean size of the microspheres could be controlled quite precisely by altering the kinetics of the reaction, such that the gel point was reached at different times following phase separation.^[39] We sought to demonstrate the phase inversion polymerization technique could be adapted to azide–alkyne cycloadditions.

2. Experimental Section

2.1. Materials and Preparation

2.1.1. PEG₄–Azide Synthesis—Unless otherwise stated, all reagents were purchased from Sigma–Aldrich. Four-arm PEG-mesylate (PEG₄-mesylate; molecular weight (MW) = 10 000 Da) was first synthesized from four-arm PEG-OH (PEG₄-OH; MW = 10 000 Da; Creative PEGWorks) by mesylating the alcohol group on PEG₄-OH with mesyl chloride. This was done by dissolving PEG₄-OH in dichloromethane (DCM), adding four equivalents of triethylamine and four equivalents of methanesulfonyl chloride while on ice, and letting it react overnight under constant stirring and nitrogen flow. After removing the salt byproduct, excess DCM was removed by using the rotovap, and the PEG₄-mesylate was precipitated out using cold diethyl ether. The product was dried under vacuum overnight to remove any remaining diethyl ether. The next step was the nucleophilic azidation of the mesylate group with sodium azide. Three equivalents of sodium azide were dissolved in dimethylformamide (DMF). PEG₄-mesylate was then dissolved in the DMF mixture and put under nitrogen and constant stirring in a hot water bath at 60 °C. The reaction was run overnight. The following day required the filtration of excess salt followed by rotovapping, diethyl ether precipitation, and drying as was done for the PEG₄-mesylate. The product was dissolved in a basic water solution with a pH between 9 and 12, and then extracted with DCM over anhydrous sodium sulfate (Na₂SO₄). A standard extraction procedure was done to extract the product into DCM. After three extractions, the Na₂SO₄ was filtered out and the process of rotovapping, diethyl ether precipitation, and drying was done as before. ¹H NMR (300 MHz, CDCl₃,): (s, 902.55H, PEG), 3.0 (s, 3H, —SO₂CH₃), 4.3 (t, 2H, —CH₂OSO₂). NMR of the product confirmed that no mesylate features remained at 3.0 ppm and 4.3 ppm (Supporting Information, Figure S1).

2.1.2. PEG₄-Alkyne Synthesis—PEG₄-mesylate was synthesized as described earlier. Four-arm PEG-amine (PEG₄-amine; MW = 10 000 Da) was then synthesized from PEG₄-mesylate by an amination reaction with ammonium hydroxide. PEG₄-mesylate was first dissolved in 400 mL of 28–30% ammonium hydroxide. The PEG₄-mesylate needed to be constantly stirred in a sealed bottle for 3–4 d. Afterwards, the bottle was uncapped to allow the ammonia to evaporate while still under constant stirring, which took 3–5 d. To collect the product, the pH of the solution was raised to 13 with sodium hydroxide (NaOH), and then extracted with DCM over anhydrous Na₂SO₄. A standard DCM extraction procedure was done to extract the PEG₄-amine into the DCM. After three extractions, the Na₂SO₄ was filtered out and the combined DCM phase was rotovapped, diethyl ether precipitated, and dried as was done for the PEG₄-mesylate. The final step was PEG₄-alkyne synthesis from PEG₄-amine. PEG₄-amine was dissolved in DCM in a beaker, and 1.5 equivalents of diisopropylcarbodiimide was added to a separate spherical flask with DCM while on ice and under nitrogen flow and constant stirring. Next, 1.5 equivalents of hydroxybenzotriazole and 1.5 equivalents of propiolic acid were added to the mixture in the flask and allowed to stir for 10 min. While waiting, three equivalents of N,N-diisopropylethylamine were added to the dissolved PEG₄-amine. Finally, this mixture was slowly added to the spherical flask, and the reaction was allowed to go for 24 h in the ice bath, under constant stirring and nitrogen gas. Following that process, the urea precipitate was filtered out, and rotovapping, diethyl ether precipitation, and drying was performed. The product was then dissolved in distilled H₂O and underwent the same extraction procedure that was done for the PEG₄-amine. Further rotovapping, diethyl ether precipitation and drying were done. ¹H NMR (300 MHz, CDCl₃,): (s, 902.55H, PEG), 2.1 (s, H, CH-). NMR spectroscopy of the product confirmed the synthesis of PEG₄-alkyne with the presence of a peak at 2.1 ppm (Supporting Information, Figure S2).

2.1.3. PEG₄-Cyclooctyne Synthesis—The four-arm PEG-cyclooctyne (PEG₄-cyclooctyne) synthesis was identical to the PEG₄-alkyne synthesis with the only difference being the use of an aza-dibenzocyclooctyne with a pendant carboxylic acid (DBCO-acid; Click Chemistry Tools) instead of propiolic acid. ¹H NMR (300 MHz, CDCl₃,): (s, 902.55H, PEG), 5.1 (d, 2H, —CH₂). NMR of the product confirmed the synthesis of PEG₄-cyclooctyne with the presence of a doublet at 5.1 ppm (Supporting Information, Figure S3).

2.1.4. Preparation of Stock Solutions of PEG₄-Azide, PEG₄-Alkyne, PEG₄-Cyclooctyne, and PEG-Dithiol—All PEG derivatives were synthesized as described earlier with the exception of linear PEG-thiol (PEG-dithiol; MW = 3400 Da; Creative PEGWorks). Stock solutions of each PEG derivative were prepared at 20% (w/v) in phosphate buffered saline (PBS) (Pierce; pH 7.4).

2.2. Procedures

2.2.1. PEG₄-Azide/PEG₄-Alkyne Hydrogel Synthesis—PEG₄-azide/PEG₄-alkyne hydrogels were formed at room temperature or 37 °C by combining stock solutions of PEG₄-azide and PEG₄-alkyne in a ratio of 1:1 with PBS, five equivalents of sodium L-ascorbate and 0.5 equivalents copper sulfate (Cu(II)SO₄). Equivalents were based on moles of azide. After at least 2 min, 0.1 M ethylenediaminetetraacetic acid (EDTA) in PBS was added to the sample to chelate the copper. Excess copper was removed by washing two more times with 0.1 M EDTA and three times with PBS. The final PEG percentage was 10% (w/v).

2.2.2. PEG₄-Azide/PEG₄-Cyclooctyne Hydrogel Synthesis—PEG₄-azide/PEG₄-cyclooctyne hydrogels were formed at room temperature or 37 °C by combining stock

solutions of PEG₄-azide and PEG₄-cyclooctyne in a ratio of 1:1 with PBS. The final PEG percentage was 10% (w/v).

2.2.3. PEG₄-Azide/PEG₄-Alkyne Microsphere Fabrication—Stock solutions of PEG₄-azide and PEG₄-alkyne were combined with PBS, Na₂SO₄, sodium L-ascorbate, and Cu(II)SO₄. The final solution was in PBS and Na₂SO₄ with five equivalents of sodium L-ascorbate and 0.5 equivalents Cu(II)SO₄ (equivalents based on moles of azide). Unless otherwise noted, the concentration of Na₂SO₄ was chosen to lower the LCST of PEG such that the PEG phase separated above room temperature but below the reaction temperature. The final PEG percentage was 2% (w/v) for the reaction with the two PEG derivatives combined in a ratio of 1:1. Cu(II)SO₄ was added last to avoid premature crosslinking. Unless otherwise noted, the reaction was performed long enough such that the microspheres formed but aggregation was kept to a minimum. To halt the reaction, EDTA in PBS was added to the sample to chelate the copper. The microspheres were then centrifuged at 14 000g for 2 min and excess supernatant was removed. Na₂SO₄ and excess copper were removed by repeating this process two more times with 0.1 M EDTA and three times with PBS.

2.2.4. PEG₄-Azide/PEG₄-Alkyne Scaffold Formation—Scaffolds were formed from the washed PEG₄-azide/PEG₄-alkyne microspheres crosslinked with PEG-dithiol. Scaffold formation was performed by combining stock solutions of PEG-dithiol with the washed microspheres in a ratio of 1 vol PEG-dithiol per 1 vol of microspheres, and then 30% (w/v) dextran ($M_r \approx 100\,000$ Da) in PBS was added to phase separate the PEG in the microspheres with 2 vol of dextran solution per 1 vol of microspheres/PEG-dithiol. The dextran/PEG microsphere solution was centrifuged at 1000g for 10 min. The thiol-yne photoaddition reaction was initiated with visible light from a xenon arc lamp (Genzyme Focal Seal LS 1000; filtered at 480–520 nm) with 10×10^{-3} M Eosin Y in PBS as the photoinitiator. The light was illuminated on the sample for at least 4 min.

2.2.5. RGD Peptide Attachment to PEG₄-Cyclooctyne—This was accomplished by reacting a cysteine-containing RGD sequence (Seq: GCGYGRGDSPG; GenScript USA Inc.) with the cyclooctyne group on PEG with a thiol-yne photoaddition reaction. RGD was added to the PEG₄-cyclooctyne in a ratio of 1:8 RGD to cyclooctyne. A PEG solution was prepared with PEG₄-cyclooctyne, RGD, and 10×10^{-3} M Eosin Y at 20% (w/v) in PBS. The process was initiated with visible light from a xenon arc lamp with Eosin Y acting as the photoinitiator and was performed for at least 4 min. A stock solution of PEG₄-cyclooctyne with RGD was then used for microsphere and scaffold fabrication.

2.2.6. Michael-Type Addition of Thiols to Cyclooctyne Assay—PEG₄-cyclooctyne was tested to determine if a Michael-type addition might occur between the azadibenzocyclooctyne and thiols. Stock solutions of PEG₄-cyclooctyne and PEG-dithiol were combined in a ratio of two thiols per cyclooctyne. Solutions were left overnight on a 37 °C heating block. Samples were checked the next day for bulk hydrogel formation.

2.2.7. PEG₄-Azide/PEG₄-Cyclooctyne Microsphere Fabrication—Stock solutions of PEG₄-azide and PEG₄-cyclooctyne (or PEG₄-cyclooctyne with RGD) were combined with PBS and Na₂SO₄. Unless otherwise noted, the concentration of Na₂SO₄ was chosen to lower the LCST of PEG such that the PEG phase separated above room temperature but below the reaction temperature. The final PEG percentage was 2% (w/v) for the reaction with the two PEG derivatives combined at a ratio of 1:1. One of the PEG derivatives was added last in the microsphere fabrication procedure to avoid premature crosslinking. Unless otherwise noted, the reaction was performed long enough such that the microspheres formed

but aggregation was kept to a minimum. At the end of the reaction, three PBS washes as previously described for the PEG₄-azide/PEG₄-alkyne microsphere fabrication were performed to remove the Na₂SO₄. The microspheres were then used immediately or diluted by 15 times their volume with PBS to slow down their reaction kinetics and stored at 4 °C.

2.2.8. PEG₄-Azide/PEG₄-Cyclooctyne Scaffold Formation—Scaffolds were formed from the washed PEG₄-azide/PEG₄-cyclooctyne microspheres. Scaffold formation was performed by adding a 30% (w/v) dextran solution to phase separate the microspheres with 2 vol of dextran solution per 1 vol of microspheres. The dextran/PEG microsphere solution was centrifuged at 1000*g* for 10 min. No crosslinker was required because residual azide and cyclooctyne groups at the surface of the microspheres reacted with each other to crosslink the microspheres.

2.2.9. Cell Attachment and Spreading Assay—50 µL bulk gels were produced from a final concentration of 5% (w/v) PEG. The solution consisted of 6.25 µL PEG₄-azide stock solution, 6.25 µL PEG₄-cyclooctyne stock solution with or without RGD, and 37.5 µL of PBS and was used to coat most of the area of a 22 mm × 22 mm glass cover slip. The solutions were spread to the edges of the cover slip to wrap the hydrogel around the cover slip and prevent them from coming off. One set of cover slips had PEG with RGD, and another set of cover slips had PEG without RGD to serve as the control. The cover slips were coated with PEG and allowed to sit for 5 min. These glass cover slips were then placed in a six-well plate, and 2 mL of endothelial growth media (EGM) (MCDB 131 media supplemented with 10 µg L⁻¹ epidermal growth factor, 10 mg L⁻¹ heparin, 1.0 mg L⁻¹ hydrocortisone, 0.2% antibiotic-antimycin, 2% fetal bovine serum, and 12 mg L⁻¹ bovine brain extract (Lonza); from Sigma except where noted). Each well was seeded with 1 × 10⁵ immortalized human aortic endothelial cells (HAEC), [41] and images were taken after 48 h using an Olympus IX70 microscope with an Olympus CPlanFl 10×/0.3 phase contrast objective.

2.2.10. Fabrication of PEG₄-Azide/PEG₄-Cyclooctyne-Microsphere-Based Scaffolds with RGD in the Presence of Cells—Following the formation and washing of PEG₄-azide/PEG₄-cyclooctyne microspheres with RGD, the microspheres were centrifuged at 14 100*g* for 12 s to form a pellet and excess PBS was removed. This was done three times to remove all excess PBS. The microspheres were then resuspended in 200 µL of PBS with 500 000 HAEC. The resuspended microspheres and the HAEC were formed into scaffolds with the addition of 30% (w/v) dextran solution as described above for the PEG₄-azide/PEG₄-cyclooctyne scaffold formation procedure. Once the scaffolds were made, excess dextran and PBS are replaced with 3 mL of EGM.

2.2.11. Fabrication of PEG₄-Azide/PEG₄-Cyclooctyne Bulk—Hydrogels with RGD in the Presence of Cells PEG₄-azide/PEG₄-cyclooctyne hydrogels with RGD were formed by combining 100 µL PEG₄-azide stock solution, 100 µL PEG₄-cyclooctyne with RGD stock solution with 500 000 HAEC resuspended in 200 µL of PBS. Once the hydrogels were made, they were washed with PBS and supplemented with 3 mL of EGM.

2.2.12. Cell Viability Assay for Scaffolds and Hydrogels—A cell viability assay was performed on the scaffolds and hydrogels that were formed in the presence of HAEC. A Live/Dead Viability/Cytotoxicity Kit (Invitrogen) was used to assess cell viability according to the manufacturer's suggested protocol. Scanning confocal microscopy was performed on the scaffolds and hydrogels with 10× (0.45 DIC L WD 4.0) and 2× (0.1 WD 8.50) objectives using a Nikon Eclipse C1/80i scanning confocal microscope, and images were analyzed

using EZ-C1 FreeViewer (Nikon Corporation). Images taken with the 10× objective were processed in MATLAB to determine percent cell viability.

2.2.13. Scaffold Analysis with Fluorescent Labeling—To image scaffolds that had been formed in the presence of cells, decellularization of the scaffolds was performed prior to labeling. Cells were lysed using deionized water. After inducing hypotonic shock for 30 min, the scaffolds were washed three times in PBS. Alexa Fluor 488-azide (Invitrogen) was used to label the remaining cyclooctyne on the microspheres that formed the PEG scaffolds. Labeling was done according to the manufacturer's suggested protocol. Scanning confocal microscopy was performed on the scaffolds with 10× and 2× objectives as described earlier.

3. Results and Discussion

We investigated the formation of microspheres and porous scaffolds in the phase-separated state using copper(I)-catalyzed and copper-free azide–alkyne cycloadditions. For the copper(I)-catalyzed reaction, PEG₄-azide was mixed with PEG₄-alkyne, which was synthesized by the reaction of PEG₄-amine with propiolic acid. Reaction of PEG₄-azide with PEG₄-alkyne to form a bulk hydrogel at 37 °C required the addition of Cu(II)SO₄ and sodium L-ascorbate (Figure 1 a); however, some reaction was noted without copper at 95 °C (results not shown). For the copper-free reaction at 37 °C, PEG₄-azide was mixed with PEG₄-cyclooctyne (Figure 1b). The latter was synthesized by reaction of PEG₄-amine with a commercially available carboxyl-derivatized aza-dibenzocyclooctyne^[42] (detailed structures and reactions shown in Figure S4 in the Supporting Information). In both the copper(I)-catalyzed and copper-free reactions, each four-arm PEG derivative served as a potential crosslink site (functionality = 4), forming highly crosslinked hydrogel networks upon reaction (Figure 1 c). Reactive groups on the PEG monomers that are not consumed during microsphere formation should still be available for subsequent addition of biologically active molecules or further crosslinking,^[40] which can be used for “bottom-up” scaffold assembly (Figure 1 c).^[10,43,44]

To produce microspheres, the clickable PEG derivatives were reacted in the phase-separated state. Small microspheres (1–10 μm) were generated in the presence of sodium sulfate for both the copper(I)-catalyzed reaction (Figure 1d) and the copper-free reaction (Figure 1e). To form these small microspheres, the concentration of sodium sulfate was chosen such that phase separation did not occur at room temperature, allowing mixing of the reagents prior to the thermally induced phase separation. With the copper(I)-catalyzed reaction, 325×10^{-3} M sodium sulfate resulted in phase separation upon heating from room temperature to 37 °C. A 2 min reaction was sufficient for microsphere formation. For the copper-free reaction, a concentration of 250×10^{-3} M sodium sulfate was needed for the formation of small microspheres. The lower concentration of sodium sulfate was required because the LCST of PEG was greatly depressed by the presence of the hydrophobic dibenzocyclooctyne on the PEG, which was similar to an effect previously observed with acrylates and vinylsulfones on PEG.^[39] Microspheres could also be formed by substituting the sodium sulfate with dextran or polyacrylamide (MW = 5×10^6 – 6×10^6 Da; Polysciences, Inc.). Both of these polymers form aqueous two-phase systems with PEG (Supporting Information, Figure S5). With PEG₄-cyclooctyne, much larger microspheres (e.g., diameters of 50 μm or greater) formed if higher sodium sulfate concentrations were used. Higher sodium sulfate concentrations caused phase separation of the PEG derivatives at room temperature, and the mixing of the reagents resulted in the formation of larger droplets, presumably by flow-induced acceleration of coalescence (Figure 1f and g). The combination of large and small microspheres has been shown to produce stronger materials than those formed from particles of uniform size,^[45] and thus may be desirable and were further examined here.

In contrast, the copper(I)-catalyzed reaction generally did not produce microspheres greater than 5 μm in diameter. A high concentration of sodium sulfate ($650 \times 10^{-3} \text{ M Na}_2\text{SO}_4$) produced only small microspheres with the copper(I)-catalyzed system (Supporting Information, Figure S6). Changes in the copper concentrations also did not affect microsphere size (Supporting Information, Figure S7), nor did increases in the reaction temperature (Supporting Information, Figure S8). This is somewhat surprising as faster reaction kinetics should result in smaller microspheres.^[39] We have seen a similar result with photopolymerizations of PEG-diacrylate in which the presence of a precipitation polymerization was suggested to occur due to the enhanced solubility of the photoinitiator in the PEG-poor phase.^[46] It is possible that the copper ions also prefer the PEG-poor phase, such that a precipitation polymerization occurs in this phase, and very little crosslinking occurs in the PEG-rich droplets.

We envision using microsphere-based scaffolds for the transplantation of vascular endothelial cells to achieve rapid vascularization following transplantation.^[47,48] The high resistance of PEG materials to protein adsorption makes it necessary to functionalize the materials with cell adhesion peptides. A cysteine-containing RGD peptide (Seq: GCGYGRGDSPG) was used in a photoactivated thiol-yne reaction with PEG₄-cyclooctyne (Figure 2a). To determine the effectiveness of the RGD attachment protocol, a thin, non-porous bulk PEG hydrogel was formed from PEG₄-azide and PEG₄-cyclooctyne with about one RGD peptide per eight cyclooctyne groups. Endothelial cells seeded on bulk hydrogels without RGD peptide were unable to spread (Figure 2b). In contrast, endothelial cells seeded on hydrogels containing RGD peptide spread quite readily after 24 h (Figure 2c). The success of attaching adhesion peptides indicates that other types of peptides and molecules can be incorporated using photoactivated thiol-yne reaction for different functions, such as scaffold degradability.

Michael-type addition of thiols to some activated alkynes is known.^[49] To determine if a Michael-type addition might occur between the aza-dibenzocyclooctyne and thiols, PEG₄-cyclooctyne was incubated with PEG-dithiol in solution at a PEG concentration of 20% (w/v). This solution did not form a gel overnight at 37 °C. However, a bulk gel formed within 4 min if Eosin Y was added to the solution and exposed to intense visible light (480–520 nm) (results not shown). This indicated that the thiol-yne reaction occurred readily, but that the Michael-type addition was insignificant.

PEG microspheres were formed and then clicked together to form porous scaffolds using reactions that should not harm living cells. Due to the potential for copper toxicity,^[50] PEG₄-azide/PEG₄-alkyne microspheres were stitched together into a scaffold using a visible light-initiated thiol-yne reaction^[22] between PEG-dithiol and residual alkyne groups in the microspheres (Supporting Information, Figure S9). This was further evidence of the specific nature of the thiol-yne reaction because microspheres formed with PEG₄-azide and PEG₄-alkyne and incubated with PEG-dithiol did not form scaffolds without photoinitiation (results not shown). Scaffolds with better handling properties were then formed by reacting PEG₄-azide/PEG₄-cyclooctyne microspheres directly with each other. We found that the crosslinking of the microspheres proceeded more efficiently in the phase-separated state,^[51] so a 500 μL mixture of microspheres and 500 000 HAEC was suspended on top of 500 μL of 30% (w/v) dextran (Figure 3a). PEG/dextran aqueous two-phase systems are widely used for cellular partitioning,^[52] and toxicity was not expected to be a concern based on previous results with a cardiomyocyte cell line.^[51] Phase separation caused the microspheres to deswell and rapidly crosslink around the cells during centrifugation for 10 min at 1000g. Following centrifugation, excess dextran and PBS were replaced with endothelial growth medium.

Structural stability, high cell viability, and a network of interconnected pores are required for the eventual vascularization of the scaffold.^[53,54] The formed scaffolds supported their own weight in solution and were able to be handled for cell culture and microscopy (Figure 3 b). Two days after scaffold formation, the viability of the HAEC within the scaffolds was calculated to be $87.8 \pm 3.5\%$ (Figure 3c). The smaller images shown to the right and bottom of Figure 3 c are stacks of cross-sections in the *y-z* plane and *x-z* plane, respectively. These demonstrate that the cells are distributed in three dimensions within the scaffolds. A control was performed with PEG₄-azide/PEG₄-cyclooctyne bulk hydrogels with RGD made in the presence of cells. After 48 h, the calculated viability of the cells had dropped to $3.5 \pm 0.8\%$. Confocal images taken of the hydrogel support these data (Supporting Information, Figure S10). Comparatively, the microsphere-based scaffold performed much better than the standard bulk hydrogel. This is believed to be a result of a superior porous network structure in the microsphere-based scaffolds.

To assess network pore structure, cells were removed from the scaffolds, and remaining cyclooctyne groups present in the scaffold were labeled with Alexa Fluor 488-azide dye. Figure 3d shows the high degree of porosity found in the scaffolds. Microspheres in some regions of Figure 3d do not appear to be connected due to the two-dimensional nature of scanning confocal microscopy. The smaller images on the right and bottom of Figure 3d demonstrate that the microspheres are in fact connected in three-dimensional space. Figure 3 d also qualitatively demonstrates the abundant availability of unreacted groups on the microspheres following microsphere and scaffold formation. Unfortunately, due to the precise nature of fabricating microspheres, the density of the available unreacted groups on the microspheres cannot be readily adjusted without adversely affecting microsphere formation. If the reaction time is decreased, then the quantity of microspheres will be reduced or no microspheres will form (Supporting Information, Figure S6a). If the reaction time between the PEG derivatives is extended, then the microspheres formed will undergo excessive aggregation, inhibiting scaffold formation later (Supporting Information, Figure S6b). Following the removal of the cells, the scaffolds could still be handled and manipulated without breaking, further illustrating the connectivity of the microspheres.

4. Conclusion

We demonstrated in this study that highly porous scaffold materials can be produced by manipulating azide–alkyne cycloadditions and phase separations. The reactions were performed in two steps: one to produce hydrogel microspheres, and another to crosslink the microspheres with each other in the presence of cells. The high viability of endothelial cells during the scaffold formation process suggests that these materials are promising candidates for in vivo vascularization strategies. In contrast to bulk hydrogels, the cells are not technically encapsulated within the hydrogel, but rather are surrounded by a matrix of microspheres. We anticipate that such a strategy will yield more tissue-like architectures than bulk hydrogels due to the highly porous nature of the materials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Clickable PEG derivatives were reacted using either: (a) copper(I)-catalyzed, or (b) strain-promoted Huisgen 1,3-dipolar cycloaddition between azides and alkynes. (c) In the presence of sodium sulfate, four-arm clickable PEG derivatives phase-separated and reacted to form highly crosslinked hydrogel microspheres. These microspheres contained residual reactive groups that allowed further crosslinking. (d) Microspheres were formed using copper(I)-catalyzed azide–alkyne cycloaddition. These microspheres were formed by inducing phase separation in 325×10^{-3} M sodium sulfate upon heating to 37 °C for 2 min. (e) Microspheres were formed using strain-promoted azide-aza-dibenzocyclooctyne cycloaddition. These microspheres were formed by inducing phase separation in 250×10^{-3} M sodium sulfate upon heating to 37 °C for 2 min. (f) and (g) Larger microspheres could be formed with the strain-promoted cycloaddition by inducing immediate phase separation at room temperature (25 °C) with 500×10^{-3} M sodium sulfate, mixing the solution by

pipetting three times, and heating to 37 °C for 2 min. Mixing in the phase-separated state resulted in the formation of much larger microspheres due to enhanced coalescence of PEG-rich domains prior to gelation.^[35]

Figure 2.

Attachment of cell adhesion peptide by thiol-yne reaction. (a) RGD peptide was added to PEG₄-cyclooctyne via a thiol-yne photoaddition, reacting the thiol on a cysteine in the RGD peptide with aza-dibenzocyclooctyne on the PEG. To demonstrate attachment of functional RGD peptide, HAEC were seeded on PEG₄-azide/PEG₄-cyclooctyne bulk hydrogels. (b) Without RGD, the HAEC were not able to spread after 24 h. (c) HAEC attached and spread on hydrogels containing RGD after 24 h.

Figure 3.

(a) To form microsphere-based scaffolds, PEG microspheres were suspended 1:1 (v/v) in 30% dextran and centrifuged at 1000g for 10 min. In 30% dextran, the PEG in the microspheres phase-separated from the dextran, causing the microspheres to deswell. This greatly enhanced reaction between the microspheres and resulted in stronger scaffolds. The scaffold formed a layer consisting of microspheres mixed with cells on top of the diluted dextran phase following centrifugation. Cells were incorporated into the scaffolds simply by combining them with the PEG microspheres in 30% dextran just prior to scaffold formation. (b) Once formed, PEG scaffolds supported their own weight and could be manipulated for routine cell culture. (c) A Live/Dead assay demonstrated $87.8\% \pm 3.5\%$ viability of HAEC 2 d after scaffold formation. (d) Labeling of the microspheres with an azide-containing dye revealed a high degree of porosity within the scaffold.