Development and optimization of a dual-photoinitiator, emulsion-based technique for rapid generation of cell-laden hydrogel microspheres

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Abstract
A growing number of clinical trials explore the use of cell-based therapies for the treatment of disease and restoration of damaged tissue; however, limited cell survival and engraftment remains a significant challenge. As the field continues to progress, microencapsulation strategies are proving to be a valuable tool for protecting and supporting these cell therapies while preserving minimally invasive delivery. This work presents a novel, dual-photoinitiator technique for encapsulation of cells within hydrogel microspheres. A desktop vortexer was used to generate an emulsion of poly(ethylene glycol) diacrylate (PEGDA) or PEG-DA-based precursor solution in mineral oil. Through an optimized combination of photoinitiators added to both the aqueous and the oil phase, rapid gelation of the suspended polymer droplets was achieved. The photoinitiator combination provided superior cross-linking consistency and greater particle yield, and required lower overall initiator concentrations compared with a single initiator system. When cells were combined with the precursor solution, these benefits translated to excellent microencapsulation yield with 60–80% viability for the tested cell types. It was further shown that the scaffold material could be modified with cell-adhesive peptides to be used as surface-seeded microcarriers, or additionally with enzymatically degradable sequences to support three-dimensional spreading, migration and long-term culture of encapsulated cells. Three cell lines relevant to neural stem cell therapies are demonstrated here, but this technology is adaptable, scalable and easy to implement with standard laboratory equipment, making it a useful tool for advancing the next generation of cell-based therapeutics.

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1. Introduction

Cell-based therapeutics have been proposed for the treatment of many diseases and conditions, including diabetes [1–9], damage resulting from myocardial infarction [10–14] and traumatic ischemic events in the brain [15–23]. These therapies generally involve the injection of a cell suspension, possibly stem cells or genetically modified cells, directly into the targeted location. Although current clinical trials are promising [24], the survival and engraftment of modified cells, directly into the targeted location. Although current microenvironment of the implantation site, which may not be ideal for maintaining viability because of the extent of tissue disease or injury, or simply because the cells are introduced to a non-native environment. Active clearance of implanted cells by the host immune response is another concern, especially when a non-autologous cell source is used. Encapsulating cell therapies within a supporting matrix can provide valuable protection during delivery, physically restrict cells to the target site, and isolate cells from a potentially hostile immune response. Additionally, this approach provides a means to control the microenvironment to which an implanted cell is initially exposed. Controlling this biochemical environment may be critical for improving implant success, since many cell types used in vivo for cell-based therapies are found within a niche or a specific microenvironment characterized by a unique combination of physical, cellular and biochemical cues that work together to maintain proper function of that cell type [27–31].

Bulk encapsulation of cells generally requires invasive implantation procedures and may be hindered by limitations on oxygen and nutrient diffusion, leading to cell death and necrosis at the core of the delivered device [32]. Polymer microparticles, however, provide a means of encapsulating cells in a controlled environment while improving transport and, importantly, maintaining the non-invasive nature of the therapy, since a suspension of cell-laden microspheres can easily pass through a syringe needle.

To date, cell microencapsulation has been primarily studied as a method of immunosolation [3–7, 33–36]. In this approach, materials are generally designed to be non-degradable and biointert in...
order to protect cells from a host immune response. By far the most common technique involves alginate-based materials that are cross-linked by ionic interactions as droplets come into contact with a cationic solution [33–35,37–39]. When the ultimate therapeu
tic goal is engraftment of the encapsulated cells, however, these alginates systems are not ideal, since they do not degrade in a controlled manner [32]. As a result, the implants may degrade too slowly, hindering engraftment, or become unstable, leaving cells without structural support. Additional concerns include batch to batch variability for naturally derived materials and possible toxicity from the polycations used during cross-linking, which may induce an inflammatory response following implantation [33,36]. Several alternative materials for cell encapsulation have been investigated, including agarose, chitosan and synthetic poly
mers such as PEGDA and polyvinylalcohol [33,34,40,41]. These can offer benefits such as reduced immunogenicity and greater mechanical stability; however, they have not been as extensively investigated [33].

Encapsulation can also be accomplished through the use of microfluidic systems to generate delivery materials with precise control over the size and characteristics of the particles [42,43]. The relatively slow rate of particle synthesis, however, makes these systems impractical for fabrication of clinically relevant volumes.

This research presents a simple but highly tunable method, using standard benchtop equipment, for microencapsulation of cells in PEG-based materials. Since the approach involves bulk generation of microparticles, it is rapid and potentially scalable for the production of larger, more clinically relevant volumes. As a base material, PEG is highly biocompatible and resists non-specific protein adsorption [44–46]. It can therefore act as a blank slate into which one can precisely incorporate bioactive factors such as peptide sequences or whole proteins [44–53]. For these studies, the polymer scaffold was modified with the fibronectin-derived RGDS adhesive sequence, as well as a metalloproteinase (MMP)-sensitive sequence to allow for enzyme-mediated degradation [51,53,54]. To generate microspheres, an emulsion of the liquid polymer solution was generated in mineral oil via vortexing. A unique combination of photoinitiators added to both the polymer and oil phases was optimized to provide rapid and complete cross-linking of the suspended droplets upon exposure to light. Finally, to demonstrate the adaptability of the system, three cell lines rele
vant to the example of neural stem cell therapies were chosen [33]. NIH-3T3 fibroblasts, MHP36 neural stem cells and bEnd.3 brain endothelial cells provided varying morphologies, adhesion preferences and MMP expression, allowing the exploration of the potential of the encapsulation system.

2. Materials and methods

Materials were purchased from Sigma unless otherwise noted.

2.1. Polymer synthesis and characterization

2.1.1. PEGDA synthesis

Poly[ethylen glycol] diacrylate (PEGDA) was synthesized by reacting 10 kDa PEG with 3 M excess acryloyl chloride and 1 M excess triethylamine in anhydrous dichloromethane under argon overnight. The reacted mixture was transferred to a separatory funnel, where 2 M K2CO3 was added at a 5 M excess to the PEG. After mixing well, the solution was allowed to separate, and the or
ganic phase was collected, dried with magnesium sulfate and fil
tered. The final, clear liquid was precipitated in cold diethyl ether, filtered and dried under vacuum.

2.1.2. PEG-RGDS synthesis

Monoacrylate PEG-RGDS was synthesized by reacting acrylate
-PEG-succinimidyld carboxymethyl (acrylate-PEG-SCM MW 3400 Da, Laysan Bio) with 0.2 M excess RGDS peptide (American Peps
tide) and 2 M excess N,N-diisopropylethylamine (DIEPA) in anhy
drous dimethyl sulfoxide (DMSO) on a rocker plate at room tem
temperature overnight. The reacted mixture was then dialyzed to remove unreacted peptide, lyophilized and characterized by gel permeation chromatography (Varian Inc.) to determine a conjugation efficiency of 87%.

2.1.3. PEG-PQ-PEG synthesis

The MMP-sensitive peptide GGPGQGWGQGK (abbreviated as PQ) [51,53,54] was synthesized with a solid phase peptide synthe
sizer (Aaptec) using standard Fmoc chemistry. The product was analyzed with matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-ToF, BrukerDaltonics). Enzyme degradable PEG-PQ-PEG was synthesized by reacting PQ peptide with 1 M excess acrylate-PEG-SCM and 3 M excess Dipea in anhydrous DMSO on a rocker plate at room temperature over
ight. The product was then dialyzed, lyophilized and character
ed by gel permeation chromatography to determine a conjugation efficiency of 92%.

2.1.4. Characterization of enzymatic degradation

Sensitivity of the PEG-PQ-PEG hydrogel material to enzyme-mediated degradation was determined by exposing cross-linked gels to a collagenase containing buffer. Since the PQ peptide con
ains a tryptophan in the sequence, the degradation of a hydrogel can be monitored by measuring the tryptophan absorbance peak (280 nm) in the surrounding solution over time [51]. To produce the gels, a hydrogel precursor solution was formed by mixing 10 wt% PEG-PQ-PEG or PEGDA in 10 mM HEPES buffered saline (HBS, pH 8.5) containing 3.4 μl mol−1 N-vinylpyrrolidone (NVP), 10 μM eosin Y photoinitiator and 1.5% v/v triethanolamine (TEOA, Fluka BioChemika). For some gels, 0.1% pluronic F68 (a surfactant used during microencapsulation) was added to the precursor solu
tion to verify that it did not interfere with the enzymatic degrada
tion. The polymer solution was then sterilized via filtration through a 0.2 μm membrane (Whatman). Droplets of this precur
sor solution (5 μl volume) were pipetted into the bottom of etha

ten-sterilized 200 μl cuvettes, cross-linked by exposure to 25 s of white light (Fiber-Lite, Dolan Jenner), and then incubated in 200 μl sterile degradation buffer (PBS containing 3.6 mM CaCl2 and 0.2 mg ml−1 NaNO3, pH 7.4) overnight. The solution was then exchanged with 200 μl fresh degradation buffer with or without 100 μg ml−1 collagenase (≥125 CDU mg−1. Type IV from Clostrid
ium histolyticum) and absorbance measurements at 280 nm were taken between 0 and 72 h with a UV spectrophotometer (Carey 5000, Varian). Degradation buffer without collagenase or hydrogel was used as reference blank.

2.2. Microsphere synthesis

A consistent protocol for attempting emulsion-based micro
sphere synthesis was maintained for all studies. In this approach, 20 μl of a hydrogel precursor solution was added to 1 ml of mineral oil in a glass test tube (13 mm by 75 mm), vortexed (Vortex Genie2, VWR) at full speed for 3 s, and exposed to light from a metal halide lamp (Dolan-Jenner) for 25 s. The lamp was modified with heat-absorbing and UV-blocking optical filters (Edmund Optics) to define an excitation range of 365–700 nm light. Light exposure was limited to 25 s, since microdroplets settle out of solution rapidly and can re
sult in phase separation beyond this time period. Cross-linked hydro
gel particles were then separated from the mineral oil by addition of 500 μl of phosphate buffered saline (PBS, pH 7.4) and centrifugation.
at 300g for 2 min. The oil layer was removed by aspiration, and the wash step was repeated with an additional 500 μl PBS. Finally, the buffer solution with resuspended microspheres was transferred to a multiwell plate for imaging and further characterization.

2.2.1. Comparison of single and dual photoinitiator approach
The synthesis protocol was first investigated with a single photoinitiator added only to the hydrogel precursor solution (10% 10 kDa PEGDA in HBS). For testing of 2,2-dimethoxy-2-phenyl acetophenone (also known as Irgacure 651, I651) initiated crosslinking, an I651 stock solution (300 mg ml⁻¹ in NVP) was added at 10 μl ml⁻¹ to the precursor solution. For testing of eosin Y initiated cross-linking, the precursor solution was supplemented with 3.4 μl ml⁻¹ NVP, 1.5% TEOA and 10 μM eosin Y. Microsphere synthesis was attempted according to protocol, and the product was analyzed for the presence of cross-linked particles. To investigate the effects of a dual combination of photoinitiators, the polymer solution was supplemented with 3.4 μl ml⁻¹ NVP, 1.5% TEOA and 10 μM eosin Y, while the oil phase was also supplemented with 3 μl ml⁻¹ of I651 stock solution. Finally, in order to isolate the effect of the oil phase photoinitiator, the synthesis protocol was conducted with 3 μl ml⁻¹ of I651 stock solution added to the oil phase and no photoinitiator in the precursor solution. All samples were imaged under brightfield microscopy with a ProgRes C5 (Jenoptik) CCD camera mounted to a Zeiss Axiovert 135 inverted microscope. For improved visualization, 0.04% trypan blue solution [55] was added, and samples were incubated for a minimum of 4 h before imaging on a dissecting microscope affixed with the ProgRes C5 CCD camera.

2.2.2. Characterization of dual photoinitiator technique
The dual photoinitiator combination for microsphere synthesis was further investigated by varying initiator concentrations and adding a cell-compatible surfactant, pluronc F68 [56]. Hydrogel precursor solution was formed by mixing 10% 10 kDa PEGDA in HBS containing 3.4 μl ml⁻¹ NVP and 1.5% TEOA both with and without 0.1% pluronc F68. Eosin Y photoinitiator was added to the solutions at final concentrations of 0, 5, 10 or 15 μM. Separately, sterile light mineral oil was supplemented with 0, 1, 3 or 5 μl ml⁻¹ of I651 stock solution. For all combinations of initiator concentrations with and without the surfactant, microspheres were generated and collected in multiwell plates according to the described protocol. To determine particle yield and size distribution, all samples were stained with 0.04% trypan blue and imaged on a dissecting microscope affixed with the ProgRes C5 CCD camera. ImageJ software was used to threshold, watershed and finally analyze the stained-particle images to produce a particle count per view field and histogram of particle diameters. For analysis of particle diameter distribution, three fields of view (>900 individual microspheres) were analyzed for each group.

2.3. Cell lines and characterization
2.3.1. Cell culture
Conditionally immortalized neural stem cells (MHP36 line, passage 56–62) [21,57] were isolated and transformed as previously described [58]. The cells were cultured on fibronectin-coated tissue culture polystyrene (TCPs) flasks, which were prepared by applying 0.07 ml cm⁻² of a 0.01 mg ml⁻¹ solution of fibronectin to the surface for 15 min at room temperature. Cells were cultured in a 1:1 mixture of Dulbecco’s Modified Eagle’s Medium and Ham’s F12 (DMEM/F12, Invitrogen) supplemented with 0.03% bovine serum albumin, 94 μg ml⁻¹ apo-transferrin, 15.3 μg ml⁻¹ putrescine dihydrochloride, 4.7 μg ml⁻¹ insulin, 380 ng ml⁻¹ l-thyroxine, 317 ng ml⁻¹ tri-iodo-l-thyronine, 1.9 mM l-glutamine (Invitrogen), 58 ng ml⁻¹ progesterone, 38 ng ml⁻¹ sodium selenite, 9.4 units ml⁻¹ heparin sodium salt, 10 ng ml⁻¹ basic-FGF (Peprotech), 1.2 ng ml⁻¹ interferon (Peprotech), 100 U ml⁻¹ penicillin (Invitrogen) and 100 μg ml⁻¹ streptomycin (Invitrogen). The cells were incubated at 33 °C in a 5% CO₂ environment. Murine brain microvascular endothelial cells (bEnd.3, ATCC, passage 25–30) were cultured in high glucose DMEM (Invitrogen) with 2 mM l-glutamine, 10% fetal bovine serum (FBS, Atlanta Biologicals), 100 U ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin at 37 °C in a 5% CO₂ environment. NIH-3T3 fibroblasts (ATCC, passage 128–135) were cultured in high glucose DMEM, with 2 mM l-glutamine, 10% bovine calf serum, 100 U ml⁻¹ penicillin, and 100 μg ml⁻¹ streptomycin at 37 °C in a 5% CO₂ environment.

2.3.2. Analysis of cellular MMP expression
Relative collagen-specific MMP expression among the three cell lines was determined by gelatin zymography of the cell culture supernatants. Cells were seeded in 48-well TCPS plates with 400 μl media per well. Seeding densities were chosen such that the cell cultures remained subconfluent after a 48 h incubation period. MHP36 and 3T3 cells were seeded at 20,000 cells cm⁻² and bEnd.3 cells were seeded at 5000 cells cm⁻². Appropriate media and culture conditions (as described above) were used for each cell type. After 48 h incubation, the media were collected and stored at −20 °C, and the cells trypsinized and counted by hemacytometer. Zymography was performed using a 10% gelatin precast Ready Gel (BioRad) according to manufacturer’s protocol. Briefly, media samples were diluted 1:1 with loading buffer (125 mM Tris–HCl, pH 6.8, 20% glycerol, 4% SDS, 0.005% Bromophenol Blue) and run at 100 V for 2 h. The gel was then soaked for 30 min each in renaturing buffer (2.5% Triton X-100 in MilliQ H₂O) and then developing buffer (10 mM Tris base, 40 mM Tris–HCl, 0.2 M NaCl, 5 mM CaCl₂, 0.02% Brij 35) at room temperature. Fresh developing buffer was then added, and the gel was incubated at 37 °C overnight with gentle agitation. The gel was stained with Coomassie blue (Sigma) for 1 h at room temperature and destained with Coomassie destain solution (methanol:acetic acid:water (5:1:4)) for 30 min at room temperature before imaging (LAS4000 Imager, Fujifilm) and analysis in ImageJ (NIH). Band intensity was normalized to cell count at the 48 h time point.

2.4. Applications for hydrogel microsphere constructs
2.4.1. Bioactive microsphere carriers
The ability of the hydrogel microspheres to function as microcarriers for surface-seeded cell culture was evaluated by incorporating the fibronectin-derived RGDS adhesive sequence. Non-degradable polymer solution (HBS with 10% PEGDA, 1.5% TEOA, 3.4 μl ml⁻¹ NVP, and 10 μM eosin Y) was prepared both with and without 0.1% pluronc F68. To an aliquot of each solution, PEG-RGDS was added at 3 mM and all solutions were sterilized via filtration through a 0.2 μm membrane. To form bulk hydrogel disks, a 10 μl droplet was sandwiched between sterile glass slides separated by 380 μm polydimethyl siloxane (PDMS) spacers. After exposure to 25 s of white light, the hydrogel disk was transferred to the well of a 24-well plate containing 500 μl sterile PBS. This process was repeated to prepare three samples for each solution. Microspheres were also formed from each of the precursor solutions using the previously described emulsion-based protocol and mineral oil containing 3 μl ml⁻¹ I651 stock solution. Particles were washed with PBS and transferred to a 24-well plate. The disks and microspheres were incubated overnight at 37 °C and, finally, the PBS was replaced with NIH-3T3 culture media. 3T3 fibroblast cells were passaged and seeded onto hydrogels at 3000 cells cm⁻² for bulk disks or 3000 cells μl⁻¹ of microspheres. After 24 h, the cultures were imaged under brightfield microscopy (Zeiss Axiovert 135). The percentage cell attachment was quantified visually across three fields of view for the seeded microspheres.
2.4.2. Microencapsulation cytocompatibility and efficiency

NIH-3T3 fibroblasts, MHP36 neural stem cells, and bEnd.3 endothelial cells were each tested independently with the microencapsulation protocol to observe the effect of encapsulation conditions on cell viability. Appropriate media and culture conditions were used for each cell type, as described in Section 2.3.1. For encapsulation, cells were trypanosized and resuspended at a final concentration of 30 × 10⁶ cells per ml in sterile-filtered, non-degradable polymer solution (HBS with 10% PEGDA, 1.5% TEOA, 3.4 μl ml⁻¹ NVP and 0.1% pluronic F68) containing 5, 10 or 15 μM eosin Y. Microsphere synthesis, using mineral oil containing 1, 3 or 5 μl ml⁻¹ 1651 stock solution, was performed according to protocol, with the exception that the 500 μl PBS washes were replaced with 1 ml media washes. The resultant microspheres with encapsulated cells were maintained as a suspension culture in multiwell plates for 24 h. To observe the viability and distribution of the encapsulated cells, samples were incubated for 30 min at room temperature in PBS containing 2 μM calcein AM and 4 μM ethidium homodimer (Live/Dead Kit, Invitrogen) and imaged by fluorescence microscopy (Zeiss Axiovert 135) or confocal microscopy (Zeiss Live/5 DuoScan). For assessment of microencapsulation efficiency, an additional encapsulation of NIH-3T3 cells was similarly performed at 15 × 10⁶ cells ml⁻¹ using the 10 μM eosin Y concentration and 3 μl ml⁻¹ 1651 stock solution. Resultant microspheres were permeabilized for 5 min in 0.5% Triton X, stained with 4 μM ethidium homodimer, and imaged to quantify the total number of encapsulated cells.

2.4.3. Microencapsulated cell culture

For long-term encapsulation studies, the non-degradable PEGDA was replaced with MMP-sensitive PEG-PQ-PEG polymer combined with PEG-RGD5 to allow cell spreading and migration within the artificial matrix. The three cell types were resuspended separately at 30 × 10⁶ cells ml⁻¹ in stable filtered polymer solution (HBS with 10% PEG-PQ-PEG, 1.5% TEOA, 3.4 μl ml⁻¹ NVP and 10 μM eosin Y) containing 3 mM PEG-RGDS and 0.1% pluronic F68. Microspheres were generated using 3 μl ml⁻¹ 1651 stock solution in the mineral oil and purified with media washes according to the described protocol. The resulting samples were cultured in their respective media type for 1 week with media exchanges every 2 days. After 7 days, nuclear and actin staining was performed to aid morphological analysis. Briefly, the cells were fixed for 30 min in 4% paraformaldehyde, permeabilized for 15 min with 0.5% Triton X-100, blocked for 2 h with 5% bovine serum albumin, then stained for 2 h in PBS with 5 μl ml⁻¹ rhodamine–phalloidin (Invitrogen) and 2 μM DAPI to allow visualization of actin and nuclei. All staining was performed at room temperature with reagents diluted in PBS and three PBS rinses following each step. After staining was completed, samples were imaged by confocal microscopy (Zeiss Live/5 DuoScan).

2.5. Statistical analysis

An unpaired two-tailed t test was performed on all data, and significance was determined when p < 0.05. The error bars in the figures depict standard deviations for the data sets.

3. Results and discussion

3.1. Hydrogel characterization

Enzymatic degradation of PEG-PQ-PEG hydrogels was characterized in a controlled study prior to cell experiments. The release of tryptophan-containing peptide fragments provides a simple measure of hydrogel degradation over time and was simply used to characterize the protease-sensitive polymer. Fig. 1 shows the degradation profile of the PEG-PQ-PEG scaffold in the presence of 100 μg ml⁻¹ type IV collagenase (MMP 2 and 9) [59] compared with two controls: degradable gel in buffer without collagenase and non-degradable PEGDA gel in collagenase solution. Solution absorbance increased only when the degradable gel was incubated in the presence of protease, indicating that hydrogel degradation is mediated by enzymatic cleavage and not a hydrolytic process. Visual inspection after a 72 h incubation confirmed that the PEG-PQ-PEG hydrogels fully degraded when in collagenase solution, while degradable samples without collagenase and non-degradable PEGDA gels remained intact. It was also confirmed that addition of 0.1% pluronic F68 surfactant to the polymer solution did not have a noticeable effect on the degradation profile. It is important to note that a high collagenase concentration was chosen for this study to allow observation of degradation within a reasonable time frame.

3.2. Microsphere synthesis

During development of the emulsion-based encapsulation technique, cytocompatibility of photoinitiators, cell sensitivity to vortex-derived shear forces and photoinitiator solubility must all be considered. For example, emulsion-based microsphere synthesis requires rapid and complete polymer cross-linking before droplets have time to coalesce, or phase separation can occur. It was anticipated that a balance would need to be achieved between the higher photoinitiator concentrations required for rapid cross-linking and the cytocompatibility of these concentrations.

3.2.1. System optimization

For initial investigations of microsphere generation, photoinitiator was added solely to the polymer precursor solution. It was found, however, that the emulsion-based approach yielded few or no microspheres with only a single photoinitiator present at concentrations suitable for cell encapsulation [60,61]. For a hydrophobic photoinitiator with low water solubility such as 1651, poor microsphere yield may be due to rapid diffusion of the initiator into the much larger oil phase before polymerization can occur. To limit photoinitiator diffusion, the hydrophilic eosin Y photoinitiator was tested. A small number of particles were generated when eosin Y was used as the sole initiator; however, these were not consistently spherical nor fully cross-linked, as shown in Fig. 2A. Specifically, a halo of diffuse material is visible around each microsphere. From these observations, a dual-photoinitiator system was conceived to limit photoinitiator diffusion, the hydrophilic eosin Y photoinitiator and the hydrophobic initiator into the much larger oil phase before polymerization can occur. To limit photoinitiator diffusion, the hydrophilic eosin Y photoinitiator was tested. A small number of particles were generated when eosin Y was used as the sole initiator; however, these were not consistently spherical nor fully cross-linked, as shown in Fig. 2A. Specifically, a halo of diffuse material is visible around each microsphere. From these observations, a dual-photoinitiator system was conceived to limit photoinitiator diffusion, the hydrophilic eosin Y photoinitiator and the hydrophobic initiator into the much larger oil phase before polymerization can occur.

Trypan blue, a stain commonly used to assess cell viability, has also been shown in the literature to be useful in staining various biomaterials including PEG-based hydrogels [55,62,63]. For this work, the dye was found to aid in the visualization of the microspheres. In Fig. 2D, the uncross-linked outer halo on the particles is visualized as an area of lighter trypan blue staining. Based on these observations, a dual-photoinitiator system was conceived to limit photoinitiator diffusion, the hydrophilic eosin Y photoinitiator and the hydrophobic initiator into the much larger oil phase before polymerization can occur. To limit photoinitiator diffusion, the hydrophilic eosin Y photoinitiator was tested. A small number of particles were generated when eosin Y was used as the sole initiator; however, these were not consistently spherical nor fully cross-linked, as shown in Fig. 2A. Specifically, a halo of diffuse material is visible around each microsphere. From these observations, a dual-photoinitiator system was conceived to limit photoinitiator diffusion, the hydrophilic eosin Y photoinitiator and the hydrophobic initiator into the much larger oil phase before polymerization can occur.

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Degradation profile for PEG-PQ-PEG hydrogel scaffolds in buffer with or without 100 µg/ml collagenase. Degradation can be observed as increasing absorbance over time due to the cleavage and release of tryptophan-containing peptides into solution. Addition of pluronic F68 was confirmed to have negligible effect on the release profile.

Representative images of microspheres formed with: (A and D) 10 µM eosin Y in polymer phase; (B and E) 10 µM eosin Y in the polymer phase with 3 µl ml⁻¹ I651 stock solution in the oil phase; and (C and F) 3 µl ml⁻¹ I651 stock solution in the oil phase only. Arrow indicates particle which has burst and is leaking internal contents. Dual combination of initiators (B and E) provides uniform spherical particles with smooth surface.

Diagram of vortex-induced emulsion encapsulation method. A polymer precursor solution containing cells (green), adhesive ligands (red), and degradable polymer (blue) is combined with mineral oil in a glass test tube. The solution is vortexed briefly during exposure to a crosslinking light source. A dual-initiator system with a hydrophilic photoinitiator in the aqueous phase and a hydrophobic initiator in the oil phase was found to be ideal for consistent particle formation. Cross-linked microspheres were isolated from the oil by addition of buffer or media solution followed by centrifugation and aspiration of the oil layer. (For interpretation of the references to colours in this figure legend, the reader is referred to the web version of this paper.)
sults it was concluded that the combination of a hydrophilic initiator in the aqueous phase with a hydrophobic initiator in the oil phase provides dramatically improved particle synthesis and cross-linking.

3.2.2. Controlling particle size and yield

Owing to the nature of the vortex-based system, a highly heterogeneous mix of particle sizes is produced. To assess this, trypan blue stained samples made with various initiator concentrations in both the oil phase and the aqueous phase were imaged and analyzed to generate a histogram of the particle diameters. As shown by the lighter bars in Fig. 4, a combination of 10 µM eosin Y in the polymer phase and 3 µl I651 stock solution in the oil phase produces microspheres ranging from <50 to >500 µm in diameter. Larger diameter particles may be more difficult to inject or could be subject to diffusional limitations and reduced cell viability at their core. Therefore, it is desirable to control or limit the size range of the microspheres that are produced. This can be accomplished by mechanical filtration of the final particle suspension, or through addition of stabilizing surfactants during synthesis. Pluronic F68 is a PEG-based, cell-compatible surfactant that is frequently used in bioreactors as a shear protectant [56]. It was found that addition of a low concentration (0.1% pluronic F68) to the polymer solution significantly reduced the average particle size (Fig. 4, darker bars). In addition to reducing particle diameter, the surfactant also significantly increased the number of microspheres produced for all encapsulations in which the oil phase photoinitiator was present. See Fig. 5 for quantification of particle yield without (A) and with (B) the surfactant included. Both of these observations are most likely caused by the stabilizing effect of the surfactant on the emulsion, which first allows smaller microspheres to be formed within the same shear environment, and then reduces the likelihood that particles will coalesce before cross-linking can occur. Fig. 5 also shows that, with surfactant present, yield generally increases as the photoinitiator concentration in either the polymer or the oil phase is increased. This follows expectations, since higher concentrations would lead to faster cross-linking and less time for polymer droplets to settle out of solution.

3.3. Applications for hydrogel microsphere constructs

Therapies based on neural stem cells are one potential application for encapsulation technologies. Within the context of this example, three cell lines were used to evaluate the dual-photoinitiator microencapsulation system. NIH-3T3 fibroblasts provided a control, stromal cell line. MHP36 neural stem cells and brain microvascular endothelial cells represented key components of the neural stem cell niche with contrasting function, cell size and morphology [64–67].

3.3.1. Characterization of cell lines

In contrast to a two-dimensional environment, a cell’s ability to spread or migrate following encapsulation within the three-dimensional (3D) hydrogel matrix will be influenced not only by integrin activation, but also by its ability to degrade the material through secretion of proteolytic enzymes [68,69]. This is a result of the small pore sizes which physically restrict cell spreading when a non-degradable polymer is used [69]. MMP 2 and 9 expression levels were therefore characterized across the three cell lines, since they will ultimately be tested within an MMP-sensitive hydrogel material. Gelatin zymography was used to determine the relative amounts of collagen-specific MMP expressed by the three cell lines. For this assay, protease activity appears as clear bands against a dark background corresponding to MMP 2 at 62 kDa and MMP 9 and at 92 kDa. Fig. 6A and B shows the zymogram results and quantified band intensity, adjusted according to the final cell number for each cell type and normalized to 3T3 MMP 2 expression. MMP 2 and 9 expression was clearly identified in the endothelial and fibroblast cell cultures. The bEnd.3 cell line, known for its enhanced proteolytic activity [70], showed over five times higher expression compared with the 3T3 cells. It was also noted, however, that the inactive form of MMP 2 could be detected for the 3T3 cell culture as a band directly above that of the active form. This cell line may therefore retain potential for enhanced collagenase activity under conditions which promote cleavage and activation of pro-MMP 2. MHP36 neural stem cells, in contrast, had no detectable collagenase activity using this assay. This data shows that the chosen cell lines provide a broad range of collagenase activity and will probably vary in their response when encapsulated within the MMP-sensitive hydrogel used in this work.

3.3.2. Microspheres as surface-seeded carriers

To determine whether the microsphere formulation provides an equivalent matrix to traditionally formed hydrogels, they were first evaluated with surface seeded cultures of the fibroblast control cell line. PEGDA hydrogels naturally resist protein adsorption and therefore do not support cell attachment [44–46]. Incorporation of adhesive peptide sequences into the matrix, however, can provide attachment sites and allow seeded cells to spread on the material [44–53]. The fibronectin-derived RGDS peptide sequence

![Fig. 4. Comparison of particle size distribution for microspheres formed with 10 µM eosin Y in the polymer phase and 3 µl I651 stock solution in the oil phase with and without 0.1% pluronic F68 additive. The presence of the surfactant shifts the particle diameter distribution to the left such that average size is reduced from 280 to 95 µm diameter.](image-url)
is known to bind cell surface integrins, stimulating adhesion and spreading on synthetic scaffolds such as PEG-based hydrogels [44,45,47,50]. In this work, the RGDS peptide was covalently linked to an acrylated PEG spacer so that it could be cross-linked into the hydrogel matrix. Bulk hydrogel disks and hydrogel microspheres were generated from non-degradable precursor material containing 0 or 3 mM PEG-RGDS. NIH-3T3 fibroblasts were then seeded and incubated for 24 h to allow time for the cells to interact with the scaffold. Fig. 7 shows that both the bulk and microsphere forms of the RGDS-containing hydrogel behaved similarly by supporting cell attachment and spreading. No cell attachment was observed for samples that did not contain RGDS. Quantification of these images confirmed 0% of cells attached to microspheres lacking PEG-RGDS, while 83% of cells were adherent on microspheres with 3 mM PEG-RGDS incorporated. These results confirm that the emulsion-based synthesis did not affect the bioactivity of the scaffold, and show the feasibility of using this technique for the generation of microcarriers.

### 3.3.3. Microencapsulation cytocompatibility and efficiency

Photoinitiator sensitivity is known to vary across cell types [61]. Therefore, by optimizing the encapsulation system across multiple lines it was expected that the chosen concentrations would be more useful for a wider range of applications. Encapsulations were performed across all combinations of a high, medium and low concentration of I651 and eosin Y. Calcein AM and ethidium homodimer staining then allowed direct observation of cell viability and distribution. Ultimately, the ideal conditions will minimize cytotoxicity while maximizing microsphere yield. As previously shown in Fig. 5, higher eosin Y and I651 concentrations generate a larger number of microspheres. Fig. 8, however, reveals significantly reduced viability in nearly all cases when I651 initiator concentration in the oil phase is increased. Although the lowest I651 concentration (1 µl stock solution per ml oil) produced the highest cell viability, it generally resulted in the formation of fewer, larger microspheres or misshapen particles. Higher concentrations of eosin Y generally did not impact cell viability for the cell lines tested, however other cell types could be more sensitive. For these reasons, intermediate levels of 10 µM eosin Y in the polymer phase and 3 µl ml⁻¹ I651 stock solution in the oil phase were chosen as the standard cell encapsulation conditions for subsequent experiments. Fig. 8D shows a representative image of MHP36 cells encapsulated under these conditions followed by labeling with calcein AM and ethidium homodimer. The results indicated that the cells are well dispersed within the spheres, and quantification confirmed viability of >80%. Finally, the efficiency of the encapsulation

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**Fig. 5.** Quantification of particle yield across a range of eosin Y and I651 concentrations both (A) without and (B) with 0.1% pluronic F68 added to the polymer phase. In general, increasing particle yield is observed with increasing photoinitiator concentrations. In addition, a significant increase in particle yield occurs upon addition of 0.1% pluronic F68 surfactant for all conditions in which I651 is present.

**Fig. 6.** (A) Gelatin zymogram and (B) quantified expression after normalization, showing varying levels of MMP expression across three cell lines: MHP36, negligible expression; 3T3, low expression; bEnd.3, high expression.
technique was assessed with NIH/3T3 cells using the standard cell encapsulation conditions. For these conditions, microencapsulation efficiency was quantified at 85% of the starting cell number.

3.3.4. Microencapsulated cell culture

For cell spreading to occur following 3D encapsulation within PEG hydrogels, the surrounding matrix must both support cell adhesion and be capable of degrading to make room for the advancing filopodia [68]. Thus, for long-term microencapsulated culture, an MMP-sensitive peptide was embedded in the backbone of the PEGDA chains to render the hydrogel sensitive to cell-secreted enzymes. This PEG-PQ-PEG polymer, in combination with the adhesive PEG-RGDS additive, can potentially provide a convincing extracellular mimic in which encapsulated cells can spread and migrate in 3D [49,52]. Individual microencapsulated cultures of these three cell lines were assessed by DAPI and rhodamine-phalloidin staining after 7 days for visualization of actin and nuclei (Fig. 9D–F). The autofluorescence from residual eosin Y photoinitiator was also used to visualize the location of the hydrogel material surrounding the cells (Fig. 9A–C). As expected, the cell lines varied in their observed ability to degrade and spread in the hydrogel. For MHP36 cells, the combination of negligible collagenase expression and a high rate of proliferation contributed to the formation of neurosphere-like clusters of cells over time. It is further indicated that these cell clusters are displacing rather than degrading the surrounding matrix based on observations of stretching and distortion of the hydrogel in the eosin Y fluorescence channel (Fig. 9A). In encapsulated 3T3 cultures, phalloidin staining revealed fine process extensions on most cells and, in some cases, clear spreading of cells within the matrix. This response is predicted by their

Fig. 7. NIH-3T3 fibroblasts seeded on the surface of (A) a bulk hydrogel disk and (B) hydrogel microspheres containing 3 mM PEG-RGDS adhesive ligand. Microsphere formulation is observed to maintain bioactivity comparable with that of the traditionally formed hydrogel.

Fig. 8. Viability analysis for (A) NIH-3T3, (B) MHP36 and (C) bEnd.3 cell lines following microencapsulation. Eosin Y photoinitiator concentration in the polymer phase was varied from 5 to 15 µM, while I651 stock solution added to the oil phase was varied from 1 to 5 µL ml⁻¹. A representative brightfield and fluorescence overlay of microencapsulated MHP36 cells stained with calcein AM (green, live) and ethidium homodimer (red, dead) is shown in (D). Variations in photoinitiator sensitivity can be observed across the three cell lines. (For interpretation of the references to colours in this figure legend, the reader is referred to the web version of this paper.)
moderate level of MMP expression as observed by zymography. The bEnd.3 cell line, in contrast, showed dramatic process extension on all encapsulated cells and extensive spreading within the 3D scaffold, which is consistent with their high expression levels of MMP 2 and 9. Furthermore, by maintaining viable cultures of all three cell lines to the 7-day time point, the ability of the degradable hydrogel microspheres to provide a conducive, 3D culture environment for a variety of cell types was demonstrated.

4. Conclusions

This research presents a novel method for simple, rapid, microencapsulation of cells using universally available laboratory equipment. In comparison with the commonly used calcium-alginate microencapsulation system, the method presented here allows for more fine-tuned control over the material’s physical and biochemical properties. Furthermore, owing to the bulk generation approach, it is potentially scalable with few additional modifications. One drawback to the vortex-based emulsion generation is the heterogeneous mix of particle sizes that is produced. Although surfactant addition and filtration can be used to select a desired particle size range, further improvement of the technique could be accomplished through the use of a more controlled shear environment for emulsion generation.

We have shown that this system can be applied to a variety of cell types and that the material can be modified for both cell adhesion and enzyme-mediated degradation. Although the integrin-binding RGDS peptide and the collagenase-sensitive PQ peptide were demonstrated in this work, the system is highly modular such that any combination of adhesive and protease cleavable sequences could be used in order to tune the polymer scaffold for a specific application. In addition, previously published work demonstrated that whole proteins can be incorporated into this hydrogel system as releasable or covalently tethered moieties [48,53]. For example, the integration of growth factors or cytokines in the microsphere formulation could provide more sophisticated control over the behavior of the encapsulated cells or could be used as a method for local drug delivery following in vivo implantation.

The flexibility and ease of microsphere synthesis as presented in this work indicate potential for both in vitro studies and in vivo cell delivery. It is expected that this approach will serve as an additional, highly useful tool for the rapid generation and study of cell-based constructs for a wide variety of applications.

Appendix A. Figures with essential color discrimination

Certain figures in this article, particularly Figures 3, 8, and 9, are difficult to interpret in black and white. The full color images can be found in the on-line version, at doi:10.1016/j.actbio.2011.06.011.

References
