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Modular Poly(Ethylene Glycol) Scaffolds Provide the Ability to Decouple the Effects of Stiffness and Protein Concentration on PC12 Cells

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Abstract

This research focused on developing a modular poly(ethylene glycol) (PEG) scaffold, assembled from PEG microgels and collagen I, to provide an environment to decouple the chemical and mechanical cues within a three dimensional scaffold. We first characterized the microgel fabrication process, examining the size, polydispersity, swelling ratio, mesh size, and storage modulus of the polymer particles. The resulting microgels had a low polydispersity, PDI=1.08, and a diameter of ~1.6 μm . The mesh size of the microgels, calculated from the swelling ratio, was 47.53 $\text{\AA}$. Modular hydrogels (modugels) were then formed by compacting EDC/NHS activated microgels with PEG-4arm-amine and 0, 1, 10, or 100 $\mu\text{g/mL}$ collagen. Stiffness (G^*) of the modugels was not significantly altered with the addition of collagen, allowing for modification of the chemical environment independent from the mechanical properties of the scaffold. PC12 cell aggregation increased in modugels as collagen concentrations increased and cell viability in modugels was improved over bulk PEG hydrogels. Overall, these results indicate that further exploration of modular scaffolds formed from microgels could allow for a better understanding of the relationship between the chemical and mechanical properties and cellular behavior.

Keywords

poly(ethylene glycol); hydrogel; modular scaffold; tissue engineering; microgel; collagen

1. Introduction

In recent years, hydrogels have demonstrated excellent results for use in tissue engineering applications, as these materials provide a water-swollen environment with chemical and mechanical properties similar to that of natural tissue [1–4]. One polymer that is currently being examined for use in tissue engineering applications is poly(ethylene glycol) (PEG), where its biocompatibility and non-immunogenicity [5, 6] has encouraged appropriate

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cellular response in neural [3, 4], cardiovascular [7, 8], and orthopedic [1, 2, 9] applications. The combination of mechanical and chemical properties exhibited by PEG hydrogels can be readily altered to obtain a desired cell response [10]. Unfortunately, the covalent attachment of proteins and peptide sequences to hydrogels has been shown to alter the chemical and mechanical properties simultaneously [4, 7, 10, 11]. This problem is particularly difficult to overcome when cells are seeded within a three dimensional (3D) material. For example, our lab recently demonstrated that neurite growth was enhanced on gels exhibiting low stiffness and increasing concentrations of laminin. However, the exact significance of the durotactic and haptotactic effects associated with these gels cannot be delineated due to the fabrication technique associated with these scaffolds, which causes the two properties to remain correlated [11]. The Leach lab has also reported evidence that traditional PEG hydrogels exhibit interrelated chemical and mechanical characteristics, where varying the concentrations of cell-adhesive peptides not only provided diverse chemical cues, but also altered the mechanical properties of the gels [10]. Therefore, while the results from traditional bulk gels are promising, it has proven difficult to decouple the chemical and mechanical cues within PEG gels using typical fabrication techniques.

To fully understand the effects of tissue engineered constructs on cell growth and tissue development, effects from the chemical and bulk mechanical cues within a 3D scaffold must be delineated. Relatively few groups have achieved isolation of a scaffold's mechanical and chemical properties. PEG-fibrinogen gels have been fabricated that utilized PEG-diacrylate (PEG-DA) as a crosslinker to alter the mechanical stiffness while maintaining a constant adhesiveness [12, 13]. While these materials offer significant advantages over gels exhibiting properties that cannot be individually modulated, the bulk of these scaffolds was fibrinogen, which provided a large number of inherent targets for cellular breakdown. To remove the aspect of uncontrolled degradation, a modular scaffold assembled from synthetic polymer microgels and protein could be utilized.

The use of polymer microgels for biomedical and pharmaceutical applications has received increased attention in recent years. These polymeric materials are characterized by their small and uniform size, large surface area, and formation of stable dispersions. Due to the improved thermal, chemical, and physical properties exhibited by microgels, these particles are currently being investigated for use in a number of applications, including controlled drug delivery [14–17] and tissue engineering applications [18–20]. The preparation of microgels formed via precipitation polymerization creates highly crosslinked microgels with relatively low polydispersity [19, 21] in an aqueous environment. Typically phase separation must be performed at high temperatures, however several groups have demonstrated that the inclusion of kosmotropic salts allows for precipitation at more physiologically relevant temperatures [21–23]. To form modugels (scaffolds formed from microgels), active molecules are added during microgel fabrication to influence the degree of crosslinking and overall scaffold stiffness [21, 24]. The inclusion of extracellular matrix (ECM) components as crosslinkers in forming the modugel then provide the chemical cues necessary for a bioactive scaffold [4].

This study investigated the ability of modular scaffolds composed of PEG microgels crosslinked via collagen I, which influences cell attachment and expression [25, 26], to encourage and support neuronal cells. We first characterized the PEG microgels including the fabrication process and the formation and physical properties of the modugels. In addition, this work examined the behavior of rat pheochromocytoma (PC12) cells, an immortalized neuronal cell line, within modular scaffolds and traditional bulk PEG scaffolds. PC12 aggregate size and overall cell viability was increased in modugels as compared to the bulk PEG gels. In both gel types, neuronal cell behavior was also improved with increased collagen concentrations. As the bulk mechanical properties of the modugels

were not impacted by the increase in collagen, the methods described herein could lead to the development of more carefully understood and constructed scaffolds where the mechanical and chemical properties are decoupled.

2. Materials and Methods

2.1 Materials

PEG-DA (Fluka, MW 3000 Da) was synthesized as previously described [2] and characterized using nuclear magnetic resonance (NMR). The acryl-PEG-glycine conjugate was fabricated by covalently binding N-hydroxysuccinimidyl group (NHS) on the acryl-PEG-NHS molecule to the terminal amine on the amino acid glycine [27]. Equal amounts of acryl-PEG-NHS (Nektar, MW 3400 Da) and glycine (Sigma) were mixed overnight at RT in PBS and stored at -20°C . Irgacure 2959 was obtained from CIBA; F12K, triethyloxylamine (TEOA), dextran (MW 67 kDa), sodium sulfate (Na_2SO_4), 2-(N-morpholino)ethanesulfonic acid hemisodium salt (MES), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), NHS, amine-modified polystyrene (PS) beads ($0.5\ \mu\text{m}$ diameter, fluorescent orange, excitation/emission of 520/540 nm), and Hoescht 33342 were obtained from Sigma; nerve growth factor (NGF, 2.5s) was obtained from BD Biosciences. Fetal bovine serum (FBS) was obtained from Biowhittaker and horse serum (HS) was purchased from Hyclone. Live/Dead staining kit was obtained from Invitrogen; PEG-4arm-amine (MW 20 kDa) was obtained from Creative PEG Works. Collagen I was isolated from rat tail tendons by dissolving tendons in 0.02 M acetic acid at 4°C , followed by subsequent centrifugation, dialysis, and storage in 0.02 M acetic acid until use, similar to previous reports [28, 29].

2.2 Microgel Fabrication

To produce PEG microgels, a solution of 20% (w/v) PEG-DA and 0.35 mg/mL acryl-PEG-glycine in PBS was heated to 37°C . Solutions of 1.5M Na_2SO_4 , 1.52% TEOA and 0.64% 6M hydrochloric acid (HCl) in PBS, and 0.5% Irgacure 2959 were heated to 37°C . The PEG solution was diluted in PBS/TEOA/HCl buffer and Irgacure (Figure 1A). Na_2SO_4 was added to the solution, such that the final solution contained 525 mM Na_2SO_4 , 2% (w/v) PEG-DA, and 0.02% Irgacure. Solutions were photopolymerized via ultraviolet light ($3.2\ \text{mW}/\text{cm}^2$, 365 nm) for thirty seconds. To remove excess materials, suspensions of microgels were buffer exchanged 3x into 100 mM MES, diluting the microsphere solutions 3:1 with MES, vortexing, and centrifuging at $4500g$ for 5 minutes to pellet the microgels. Microgels were rinsed 5x in DI H_2O , lyophilized to dryness, and resuspended at a mass of 27.2 mg per mL of 100 mM MES. Yield of microgels was found after each reaction from dried samples.

2.3 Microgel Characterization

2.3.1 Microgel Size—Differential interference contrast (DIC) microscopy was used to determine the size of the PEG microgels. Microgel samples were imaged using a Zeiss inverted microscope with 100x/oil DIC objective and Axiovision software was utilized to determine size. A sample size of $N > 1000$ microgels was utilized to determine average particle size. The polydispersity of the microgels was calculated as:

$$PDI = \frac{\frac{\sum V_i^2}{\sum V_i}}{\frac{\sum V_i}{N}}$$

Eqn 1

where V_i is the volume of microsphere i and N is the total number of microgels [21]. The size of the microgels was also measured using dynamic light scattering (DLS; 90Plus

Particle Size Analyzer, Brookhaven Instruments) at a scattering angle of 90° and wavelength of 658 nm. Disposable polystyrene cuvettes were cleaned 1x with 95% ethanol and 3x with DI H₂O prior to use. To measure the size of the microgels, 15 µL of the microgel stock solution was diluted in 3 mL of PBS and analyzed at 25°C. Calculation of the mean effective diameter and statistical analysis of the results were performed using Brookhaven Instruments Particle Sizing Software.

2.3.2 Microgel Density—Isopycnic density gradient centrifugation was utilized to determine the density of the microgels. Dextran was dissolved in DI H₂O to form 1, 5, 10, 15, and 20% (w/v) dextran solutions, which were carefully layered in a centrifuge tube. A sample of microgel stock solution was placed either above the 1% layer or below the 20% layer; the gradient was centrifuged for 10 minutes at 4000g. As the density of the dextran solutions is easily calculated, the density of the microgels can be estimated based on their relative position post-centrifugation. Density was determined using N=5 samples.

2.3.3 Microgel Swelling Experiments and Mesh Size Calculations—To estimate the swelling ratio, 500 µL of stock microgel solution was centrifuged and the supernatant removed. To remove excess buffer salts, microgels were rinsed five times in DI H₂O. After rinsing, the microparticles were centrifuged, the supernatant was removed, and the mass after swelling (M_s) was measured. The microgels were then lyophilized to dryness and their dry mass (M_D) was measured. The swelling ratio based on microgel mass (Q_M) was calculated as [30]:

$$Q_M = \frac{M_s}{M_D} \quad \text{Eqn 2}$$

The swelling ratio was observed for N=5 samples. Q_M was further used to calculate the volume swelling ratio (Q_V):

$$Q_V = 1 + \frac{\rho_p}{\rho_s}(Q_M - 1) \quad \text{Eqn 3}$$

where ρ_p is the density of the dry microgel and ρ_s is the density of the solvent. The molecular weight between entanglements (M_e) could then be calculated as [31]:

$$\frac{1}{M_e} = \frac{2}{M_n} - \frac{\frac{v}{V_1}(\ln(1 - v_2) + v_2 + x_1 v_2^2)}{v_2^{1/3} - \frac{v_2}{2}} \quad \text{Eqn 4}$$

where M_n is the number average molecular weight of the uncrosslinked macromer, V_1 is the molar volume of the solvent, v_2 is the polymer volume fraction in the equilibrium swollen microgel, v is the specific volume of the polymer (ρ_s/ρ_p), and x_1 is the polymer-solvent interaction parameter [30, 31]. Microgel mesh size (ξ) was calculated as described previously [32]. The root-mean-square of the end-to-end distance of the polymer chain in the unperturbed state [$(r_o^2)/2$] determined as:

$$(r_o^2)^{1/2} = l C_n^{1/2} n^{1/2} \quad \text{Eqn 5}$$

where l is the average bond length, C_n is the characteristic ratio of the polymer PEG, and n is the number of bonds in the crosslink [30]:

$$n = 2 \frac{M_e}{M_o} \quad \text{Eqn 6}$$

where M_o is the molecular weight of the repeat unit. The mesh size was then calculated as:

$$\xi = v^{-1/3} (r_o^2)^{1/2}. \quad \text{Eqn 7}$$

From the mesh size, the storage modulus, G' , of the microgels could then be calculated as [33]:

$$G' = \frac{\rho RT}{M_e} \quad \text{Eqn 8}$$

2.3.4 Activation of PEG-glycine—Microgels were EDC/NHS activated, as described in a previous protocol [21], adding 27.8 mM EDC and 50.1 mM NHS in 10 mM MES buffer solution (pH 4) for every 27.3 mg of microgels. After mixing for 30 min (Figure 1B), activated microgels were collected via centrifugation and mixed with PBS, containing amine-modified PS beads, overnight on a rotator at room temperature. The microgel-PS bead solutions were washed with PBS to remove unbound beads. To observe conjugation, samples were diluted 1:10 with PBS, sonicated for 2 min, and imaged on a Zeiss inverted microscope with a 100x/oil DIC objective. Conjugation was observed in N=5 samples.

2.4 Modugel Assembly

After EDC/NHS activation, as described above, activated microgels, PEG-4arm-amine (1:1 PEG-glycine:PEG-4arm-amine), and 0, 1, 10, or 100 $\mu\text{g/mL}$ collagen I were combined and briefly mixed in PBS (Figure 1C). Solutions of microgels, PEG-4arm-amine, and collagen were placed into wells and centrifuged at 2254g for 20 min. The supernatant was collected after compaction to examine collagen content via electrophoresis. Modugels were incubated at 37°C overnight, allowing gels to further crosslink. After crosslinking, several gels were stained with eosin to examine the collagen content.

2.5 Mechanical Characterization of Modugels

Modugels, fabricated as described above, were formed in 4 cm^2 silicone wells. Using a die, the gels were punched into 8 mm rounds, each approximately 1 mm in height. The mechanical stiffness of each gel (G^*) was measured via oscillatory shear rheometry, using an 8 mm parallel plate configuration, a frequency sweep of 1–100 rad/s, and constant strain of 5%. The results (G^*) were averaged and compared at 10 rad/s, which was within the linear viscoelastic region for all gels tested, where N=5 for each modugel type.

2.6 PC12 Cell Behavior

2.6.1 Modugels—A base layer of modugel, consisting of activated microgels, PEG-4arm-amine, 0, 1, 10, or 100 $\mu\text{g/mL}$ collagen mixed in F12K, which replaced the PBS used in previous mechanical characterization studies, was formed in 48 well plates by centrifuging solutions at 2254g for 20 minutes. Three hours post-centrifugation, the modugel supernatant was removed. A second layer of modugel solution, containing 4×10^5 PC12 (ATCC, P4–6) cells per mL of microgels, was added atop the base layer and the compaction process was repeated.

2.6.2 Bulk PEG Gels—PC12 cells were encapsulated within low concentration, bulk PEG gels, fabricated as previously described [4]. Briefly, solutions containing 3 and 4% (w/w) PEG-DA, 0 or 100 µg/mL PEG-collagen conjugate, 0.05% Irgacure 2529, and F12K were mixed PC12 cells, at a concentration of 4×10^5 cells/mL. Solutions were crosslinked under UV light (3.2 mW/cm^2 , 365 nm) for 9 mins.

2.6.3 Collagen Gels—As a control for cell aggregation and viability, PC12 cells were cultured in 1 mg/mL collagen gels, fabricated as previously described [34, 35]. A thin layer of collagen gel solution was added to the bottom of the well and allowed to gel for 30 minutes. A second layer of collagen gel solution, containing 4×10^5 PC12 cells/mL, was pipetted atop the base layer.

2.6.4 PC12 Experiments—After forming each gel, F12K supplemented with 15% HS, 2.5% FBS, and 50 ng/mL NGF, resulting in 25 ng/mL NGF in the system, was added atop the gels. Cells were cultured at 37°C, with 5% CO₂, for up to 12 days; fresh media supplemented with sera and 50 ng/mL NGF was exchanged every 2 days. Cells were nuclei stained with Hoechst 33342 and live imaged at 2, 4, 6, 8, and 12 days post-seeding, using a Zeiss inverted microscope with a 20x objective. For viability assays, 200 µL of 10 mM ethidium homodimer-1 in PBS was added to gels; gels were incubated at 37°C for two hours prior to imaging. Axiovision software was utilized to measure the size of the PC12 aggregates using fluorescent microscopy, where aggregate size was calculated as a 2D projection of aggregate, similar to previous studies [35]. The number of aggregates measured was >62 for aggregation studies; N>850 cells for viability experiments.

2.8 Statistical Analysis

Results are expressed as mean $\pm$ standard error of the mean. Statistical analysis was performed using SAS software (SAS Institute). With the exception of the PC12 cell aggregation results, all results were analyzed using ANOVA. As the Shapiro-Wilks test showed non-normal distribution for PC12 cell aggregation, nonparametric tests were used to determine statistical differences for these results. Comparisons of data representing multiple measurements over time were analyzed using Friedman's test. Quantitative values were compared by Kruskal-Wallis test. All post hoc analysis was performed using the Tukey HSD test and the threshold for statistical significance was set at $p < 0.05$.

3. Results

3.1 Microgel Characterization

3.1.1 Microgel Size—Microgels were formed from solutions containing PEG-DA and acryl-PEG-glycine with the addition of aqueous sodium sulfate at 37°C. Upon drying, it was determined that only $7.3 \pm 1.2\%$ of the original PEG-DA was incorporated into the microgels. Using optical microscopy, the average diameter of the microgels was 1.59 ± 0.14 µm, with an overall size distribution of 1.15–2.01 µm (Figure 2A); the polydispersity was calculated to be 1.08. Microgel size was confirmed using DLS, where results indicated that the average diameter of the microgels was 1.63 ± 0.13 µm.

3.1.2 Microgel Density—The density of the PEG-glycine microgels was determined via a dextran gradient. Post-centrifugation, the microgels were located between the 5 and 10% dextran layers; this relative location was independent of the original placement of the microgels. To determine a more precise density range, a gradient was formed using 1, 5, 6, 7, 8, 9, and 10% dextran solutions. Samples of microgels were placed either above the 1% layer or below the 10% layer. Upon centrifuging, microgels were positioned between the 5 and 6% dextran layers, regardless of their original placement. The density of 5% dextran

was calculated to be 1.018 g/cm³ (also reported in literature [36]), while the 6% dextran was found to have a density of 1.020 g/cm³, indicating that these PEG microgels have a density between 1.018–1.020 g/cm³.

3.1.3 Microgel Swelling Experiments and Mesh Size Calculations—The microgel network composition was probed by examining the swelling capacity and the mesh size of the hydrogel particles. Upon completing microgel swelling experiments, Q_M of the PEG microgels was calculated to be 10.3. As the concept of Q_M is related to ξ , the mesh size of the PEG microgels was found to be 47.53 Å, with an average molecular weight between entanglements (M_e) of 1253.49 Da. Using M_e , an estimate for the microgel storage modulus, G' , was calculated to be 461 kPa.

3.1.4 Activation of PEG-glycine—EDC/NHS activated microgels were mixed with aminated PS beads overnight. After sonicating and rinsing microgels, samples were imaged in order to confirm successful attachment of aminated beads to microgels (Figure 2B). The PS beads were visible only directly adjacent to microgels, indicating the presence of PEG-glycine functional groups on the surface of the polymer particle. Notably, aggregates of activated microgels and aminated PS beads were visible even after extensive sonication. Multiple functional groups are available on the surface of each PS bead, enabling the aminated beads to bind to several microgels and serving as crosslinking agents to create microgel aggregates. The formation of aggregates is important as it indicates that crosslinking materials can be combined with activated microgels to form modugels.

3.2 Examination of Modugels

The supernatant was collected after modugel compaction for analysis via electrophoresis. For the 100 µg/ml collagen-modugel samples, no protein bands were detected, demonstrating that at least 96% of the collagen remained with the microgels upon compaction. In addition, the modugels were labeled with Eosin and collagen-containing modugels were visually pink throughout in comparison to the non-collagen containing controls.

Rheological testing was utilized to determine G^* , a measure of mechanical stiffness, of the formed modugel scaffold. As expected for these materials, the storage modulus (G') was greater than the loss modulus (G''); thus, all scaffolds were considered gels. All modugels were compressed within the parallel plates to a constant value (26.7 Pa), which provided more consistent analysis for the hydrogel material. Modugels, fabricated without the addition of collagen, exhibited an average stiffness of 120.78 ± 2.31 Pa. The stiffness exhibited by the gels was not significantly altered with the addition of collagen at any concentration (Table 1).

3.3 PC12 Cell Behavior

3.3.1 Aggregation—PC12 cells were suspended within the scaffolds and aggregated cells were visible in all gels after two days of culture. Cells were found throughout the top layer of the modugel (Figure 3). Due to centrifugation slightly more cell aggregates were found in the lower portion of the top layer than in the higher portion; however, all cells were suspended within the 3D matrix due to the prior application of the bottom modugel layer. Modugels fabricated without collagen exhibited an average aggregate size of 1070.48 ± 64.07 µm² on day 2 (Figure 4). Similar sized aggregates were visible in bulk PEG gels with no collagen, where the average size was 1110.60 ± 37.93 and 997.23 ± 43.49 µm² for 3 and 4% PEG gels, respectively. The addition of 100 µg/mL PEG-collagen to either the 3 or 4% PEG gels resulted in a significant increase in cell aggregation. Likewise, as the concentration of collagen increased within the modugels, aggregate size also significantly

increased, where modugels containing 100 $\mu\text{g/mL}$ collagen exhibited aggregates of $1669.96 \pm 140.27 \mu\text{m}^2$ after only two days of culture. For comparison, PC12 aggregation in 1 mg/mL collagen gels was examined. Aggregates were found to be significantly larger in the collagen gels compared to all bulk gels, as well as modugels containing 0 and 1 $\mu\text{g/mL}$ collagen, where the average size was $1848.05 \pm 100.73 \mu\text{m}^2$ in collagen gels on day 2.

In order to examine the modugels' capabilities of supporting cell growth over time, gels were cultured over a period of 12 days. No significant change in aggregate size for modugels containing 0 $\mu\text{g/mL}$ collagen was measured over the duration of culture; however, the size of aggregates within the modugels containing 1, 10, or 100 $\mu\text{g/mL}$ collagen significantly increased with extended culture time. The size of the aggregates in 3 and 4% PEG gels with 100 $\mu\text{g/mL}$ PEG-collagen also significantly increased with extended culture time; however, the size of aggregates in plain 3 and 4% PEG gels remained similar over time. In addition, aggregation in bulk gels was generally smaller than the aggregation found in modugels. Aggregation was also found to significantly increase in collagen gels over time, where at each time point, cell aggregates within collagen gels were found to be larger as compared to aggregates in the bulk gels or modugels containing 0 or 1 $\mu\text{g/mL}$ collagen. However, modugels containing 10 or 100 $\mu\text{g/mL}$ collagen were found to have similarly sized aggregates as 1 mg/mL collagen gel controls for all time points except on day 12.

3.3.2 Cell Viability—To further examine the response of PC12 cells in the various gel types, a cell viability assay was completed. After two days of culture, collagen gels had the highest cell viability of all gel types, where, on average, $96.0 \pm 0.8\%$ of the cells were living (Table 1). Similar viability was seen in modugels with 100 $\mu\text{g/mL}$ collagen, where $93.6 \pm 0.6\%$ of cells were viable. As the amount of collagen within the modugels decreased, a corresponding decrease in cell viability was found, where only $89.9 \pm 1.1\%$ of cells were viable in modugels with 0 $\mu\text{g/mL}$ collagen. Bulk 3 and 4% PEG gels exhibited a significant decrease in the number of viable cells compared to both collagen gels and modugels containing 10 and 100 $\mu\text{g/mL}$ collagen. Cell viability was similar in bulk gels with decreasing concentrations of collagen, where 3% PEG gels with 100 and 0 $\mu\text{g/mL}$ PEG-collagen exhibited $87.4 \pm 0.8\%$ and $86.0 \pm 0.9\%$ viable cells, respectively. Cell viability decreased slightly from day 2 to day 4 in all gel types, however this decrease was not found to be significant.

4. Discussion

In the development of tissue engineered scaffolds, the use of a 3D synthetic scaffold provides mechanical and chemical properties similar to that of native tissues. While the properties of bulk PEG scaffolds can be readily altered to encourage cell growth [2–5, 7, 9], it is difficult to decouple the chemical and mechanical characteristics of the gel in 3D, as varying either of the properties unintentionally results in changes of the other. This phenomena is particularly true in lower stiffness gels ($G^* < 1 \text{ kPa}$), where the shortening of the chain that occurs with the addition of protein conjugates decreases the bulk stiffness of the gel [4]. Therefore, this work investigated scaffolds formed from PEG microgels, which would provide the blank slate of a bulk PEG hydrogel while incorporating bioactive factors to aid in crosslinking into a modugel. In this system, the mechanical properties were effectively decoupled while the amount of protein that was incorporated varied, altering the chemical properties.

The modular scaffolds in this study utilized small polymeric hydrogel particles, or microgels, as building blocks. Using both DIC microscopy and DLS, the average diameter of the PEG microgels was found to be approximately 1.6 μm . As the polydispersity of the particles was relatively low, $\text{PDI} = 1.08$, these microgels were fabricated within a very narrow

size range, agreeing with results seen previously in literature [21, 37, 38]. However, the efficiency of the microgel formation at 37°C was low, with only 7.3% of the original PEG was incorporated into the microgels. Previous studies have shown that the amount of PEG that precipitates to form the microgels can be increased by increasing the concentration of PEG in the polymerizing solution [21] or by modifying the reaction conditions, such as pH or temperature. Using the data obtained from microgel swelling experiments, M_e of the PEG microgels was found to be less than the molecular weight of the original PEG macromer (3000 Da), indicating that the entangled polymer chains were very tightly compacted. Similar to the microgels formed in this study, Marek et. al. reported that microparticles formed using a poly(diethylamino)ethylmethacrylate-co-PEG copolymer (MW 2000 Da) had a mesh size of 68 Å and a swelling ratio of 11, in neutral pH solution [39]. As these microgels are essentially small PEG hydrogels, the mesh size of the microgels was calculated using polymer theory and compared to the bulk PEG literature. The Mahoney lab demonstrated mesh sizes and swelling ratios from 20% PEG (MW 4600 Da) gels [40] that were similar to those found for the PEG microgels. In addition, the G' calculated for the microgels fabricated in this study was equivalent to the compressive modulus exhibited by 20% PEG (MW 3000 Da) gels, where the modulus was determined to be approximately 500 kPa [41]. Therefore, using this protocol, small, compact microgels with low polydispersity were formed and these microgels had similar characteristics to bulk PEG hydrogels of approximately 20% PEG.

To provide active sites for crosslinking, PEG-glycine was incorporated into the microgels. The presence of glycine moieties on the surface, and therefore availability for activation, was confirmed visually by the conjugation of NHS-activated microgels to aminated PS beads. The addition of co-monomers allowed for the functionalization of the microgels, such that they can be tailored to suit a specific application [42]. Previously, the fabrication of PEG microgels formed using various surface moieties has allowed for the creation of non-degradable [21, 38], degradable [18], and drug loaded microgels [18], demonstrating that the function of these materials is readily altered with a change of the active molecules. In addition, the use of reactive functional groups can serve as a means of crosslinking the microgels to form a modular scaffold [18, 21]. For the purposes of this study, the glycine acted as a functional group on the PEG microgels. These groups were activated and the microgels were conjugated together by binding amine groups on multi-arm PEG-amine and collagen I, in order to create a modugel suitable for tissue engineering applications.

While natural tissues and tissue engineered scaffolds exhibit an overall bulk stiffness, evidence shows that cells may sense the underlying substrate stiffness and respond based on cues from individual biomolecules [43]. Elastic moduli for neural tissue ranges from 0.1–1 kPa [44]; however, the mechanical characteristics of the individual components, such as the microgels or collagen fibers, that comprise the microscale of the material should also be considered. The stiffness of pure collagen fibrils has been reported to be 2 GPa [43], approximately 4 orders of magnitude larger than the stiffness of the microgels (~500 kPa), representing a range of microscale mechanical properties for the PC12 cells. However, previous work has demonstrated that cells can deform fibers with stiffnesses of 5–38 MPa [43]. Therefore, forces applied by migrating PC12 cells in this study should be sufficient to deform the microgel structure. As very few research groups have investigated the microscale properties of tissue engineered constructs, this research focused on comparing cell response to overall mechanical properties exhibited by modugels and bulk scaffolds.

Mechanical evaluation of the PEG modugels demonstrated that the stiffness of this 3D scaffold was between 1 mg/mL collagen gels [34] and 3 and 4% bulk PEG scaffolds [4]. The collagen gels utilized in this study exhibited a stiffness approximately one order of magnitude lower than the bulk PEG scaffolds, collagen gels were used as the control in

order to compare our results to the only 3D PC12 study found in literature [35]. Previously, this lab has shown that the addition of a protein conjugate to bulk PEG hydrogels results in a significant decrease in gel stiffness, with even 1 $\mu\text{g/mL}$ of protein conjugate [4]. For a bulk hydrogel, a decrease in gel stiffness during protein conjugate addition can be attributed to the capping of the growing polymer chain, effectively ending the chain lengthening process [4, 10]. However, the addition of protein to the modugel does not cause a physical change to the PEG chain structure; rather, the protein serves as a crosslinking agent for the overall construct [24]. In this study, the mechanical stiffness of PEG modugels was not significantly altered with increasing collagen through addition of a supplemental crosslinker (4-arm PEG-amine). The fabrication of a 3D material that maintains its mechanical properties while its chemical properties are altered is an exciting possibility for better delineating the effects of mechanical and chemical cues of a scaffold on cell behavior *in vitro*.

To examine the impact of decoupling the bulk mechanical and chemical cues within a 3D environment, PC12 cell aggregation and viability were examined in modugels, bulk PEG gels, and collagen gels. In general, PC12 cell aggregation and viability were improved in collagen gels as compared to both bulk PEG gels and modugels. A general trend was seen in the modugels and bulk PEG gels where aggregate size increased with increasing amounts of collagen, indicating that the amount of collagen encapsulated was increased within the scaffolds as the protein addition increased. The results from the electrophoresis and eosin staining, as well as the results of dependence of collagen concentration on PC12 aggregation, suggest that the collagen was retained within the gels. The influence of collagen on cell behavior is not a new discovery; research groups have been investigating the effects of ECM components on cell behavior for over a decade [1, 12, 45, 46]. Mahoney and Anseth previously reported that the size of neural precursor cell aggregates, encapsulated within degradable PEG-PLA hydrogels, increased in cultures containing collagen over those cultures lacking protein, similar to results obtained from this current study [45]. Cell viability within gels was investigated to determine the impact of gel fabrication techniques. Overall cell viability in modugels was found to be approximately 90%, even in gels formed without collagen, similar to PEG gels formed using Michael-type addition where approximately 90% of 3T3/balb fibroblasts were found viable after 24 hours [47]. For PC12 cells, aggregation and viability was generally improved in modugels over bulk PEG gels. As both gel types were fabricated using the same base materials, this result may be related to the fabrication techniques associated with each scaffold type. The exposure to free radicals generated during the crosslinking process of bulk PEG scaffolds could jeopardize the integrity of the encapsulated cells. Recently, the Khademhosseini group reported that the viability of NIH-3T3 mouse embryonic fibroblast cells decreased to 80% after 40 secs of UV irradiation, using wavelengths similar to those used for this study (360–480 nm) [48]. While the duration of UV exposure in this study was longer (9 min) compared to the previous study, similar viability (< 90%) was found for PC12 cells encapsulated in bulk PEG gels. This phenomena may be due to the difference in photoinitiator concentrations, where a 10-fold increase in Irgacure 2959 concentration was utilized in the Khademhosseini study [48], as larger amounts of photoinitiator have been shown to impact cell survival due to the creation of more free radicals [49]. In addition, LY-R cell viability was shown to decrease to approximately 50% after exposure to UVA irradiation, wavelengths of 340–400 nm, for 1 hr [50] and other groups have investigated the toxic effects of UV light on cells cultured *in vitro*, reporting that exposure to UV radiation triggers a pathway of cell death [51–53]. Unfortunately, many studies do not report the intensity of the UV light on the cells. This point would be critical to the amount of UV irradiation the cells are receiving and will be a contributing factor to cell death. Overall, our results, combined with previous reports in literature, suggest that cell exposure to free radicals and UV irradiation should be minimized. As the modugels do not require UV light to crosslink, they provide an alternative means of scaffold fabrication for tissue engineering applications.

In addition to the enhanced cellular viability exhibited in modugels, the chemical and mechanical properties exhibited by this gel type can be readily decoupled. Aggregation was shown to increase with increasing collagen concentrations within modugels exhibiting similar stiffness (Figure 5). In bulk PEG gels, aggregation was also found to increase with added PEG-collagen conjugate; however, the incorporation of the collagen conjugate not only caused a change in the chemical properties of the scaffold, but also significantly altered gel stiffness, making it difficult to delineate between effects of the scaffold's properties. By decoupling the bulk chemical and mechanical cues of the modugel, it was determined that neural cell behavior was significantly affected by the concentration of protein, where increases in collagen concentration led to increases in aggregate size. To further examine the individual effects of the scaffold, the mechanical stiffness of the modugel could be tuned by varying the amount of functional groups on the microgels [24]. The addition of functional groups to the microgels allows for further crosslinking, thus producing an overall stiffer scaffold. Similarly, a reduction in the number of functional groups would result in less crosslinking and a more flexible construct.

As the addition of NGF has been shown to suppress PC12 proliferation [35], it is expected that the increasing size of the aggregates is due to cell mobility through the constructs, triggered by environmental factors. The stiffness of the modugels in these experiments was not found to be significantly different, indicating that cell mobility is likely affected by chemical factors, such as the addition of cell-adhesion collagen molecules. Furthermore, proteolytic breakdown of the matrix could also account for enhanced cell mobility. However, as the ability to utilize modugels as scaffolds that decouple chemical and mechanical factors was limited to early time points for these *in vitro* studies, the effect of modugel biodegradation was not explored, but remains a point of interest for future studies.

The use of modular scaffolds for tissue engineering applications is very exciting. The modugels examined in this study are unique as they allow for the chemical and mechanical properties of the scaffold to be decoupled, which is not typically achieved using traditional fabrication techniques. The stiffness of the modugels, similar to that of other scaffolds currently being examined for neural tissue engineering constructs was not significantly altered with the addition of collagen at any concentration. In addition to the isolation of the modular scaffolds properties, PEG modugels exhibited improved neural cell response over bulk PEG gels. These results further encourage the exploration of modular scaffolds for tissue engineering applications.

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Figure 1. Schematic representation of modugel formation (not to scale). (A) Microgels were fabricated via precipitation polymerization using PEG-DA and PEG-glycine monomers. (B) Microgels were EDC/NHS activated and (C) mixed with PEG-4arm-amine and 0, 1, 10, or 100 $\mu\text{g/mL}$ collagen prior to centrifugation to form the modugel.

Figure 2.

(A) Histogram for PEG-glycine microgels with average diameter of $1.59 \pm 0.14 \mu\text{m}$ and size range of $1.15\text{--}2.01 \mu\text{m}$. Inset image shows PEG-glycine microgels; $N > 1000$ microgels. (B) (i) Phase and (ii) UV images of activated microgels bound to aminated PS beads. The microgels are more aggregated than in the inset image above due to the effective crosslinking by the aminated PS beads. Scale bars are $20 \mu\text{m}$.

Figure 3.

Representative images of nuclei labeling of aggregates within the collagen gel (A) and modugels with 100 $\mu\text{g/ml}$ (B), 10 $\mu\text{g/ml}$ (C), 1 $\mu\text{g/ml}$ (D), and 0 $\mu\text{g/ml}$ (E) added collagen. The two dimensional projection of the aggregates was measured within the 3D gels of those nuclei in focus. These images are from day 4; cells and aggregates are distributed throughout the gel. Scale bars are 100 μm .

Figure 4. Examination of PC12 cells in modugels, collagen gels, and bulk PEG gels demonstrated increased aggregate size with increased collagen concentration and culture time. Stars represent significance from similar gels on day 2; error bars represent standard error. N>62 aggregates.

Figure 5. Examination of PC12 aggregation (day 4) compared to scaffold stiffness for modugels and bulk PEG gels. Aggregation in bulk gels was shown to be a factor of both the chemical and mechanical cues of the scaffold; aggregation in modugels (encompassed by circle) was dependent on the gels' chemical properties. Error bars represent standard error.

Table 1

Summary of stiffness and cell viability in modugels, bulk PEG gels, and collagen gels.

Collagen Concentration($\mu\text{g/mL}$)	G^* (Pa)	Cell Viability (%)	
		Day 2	Day 4
Modugel			
0	120.4 ± 2.3	$89.9 \pm 1.1^*$	$87.4 \pm 0.9^*$
1	122.2 ± 2.7	90.8 ± 0.8	89.2 ± 0.8
10	126.3 ± 3.0	92.9 ± 0.5	90.1 ± 0.9
100	130.7 ± 4.5	93.6 ± 0.6	92.5 ± 0.7
3% PEG Gel			
0	$69.2 \pm 4.3^{[4]}$	$86.0 \pm 0.9^*$	$84.6 \pm 1.3^*$
100	$45.7 \pm 2.8^{[4]}$	$87.4 \pm 0.8^*$	$86.6 \pm 1.4^*$
4% PEG Gel			
0	$480.0 \pm 9.8^{[4]}$	$83.1 \pm 2.7^*$	$81.4 \pm 1.4^*$
100	$232.1 \pm 22.7^{[4]}$	$86.0 \pm 1.0^*$	$84.5 \pm 1.9^*$
Collagen Gel			
1000	$7.0 \pm 0.3^{[34]}$	96.0 ± 0.8	94.7 ± 0.7

* represents statistical difference in viability from collagen gels. N>850 cells.