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Neural stem cell encapsulation and differentiation in strain promoted crosslinked polyethylene glycol-based hydrogels

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Abstract

Encapsulated cell viability within crosslinked hydrogels is a critical factor to consider in regenerative medicine/cell delivery applications. Herein, a ‘click’ hydrogel system is presented encompassing 4-dibenzocyclooctynol (DIBO) functionalized polyethylene glycol (PEG), a four arm PEG tetraazide crosslinker, tethered native protein attachment ligands (laminin) and a tethered potent neurogenic differentiation factor (interferon- γ). With this approach, hydrogel formation occurs via strain-promoted, metal-free, azide-alkyne cycloaddition in an aqueous buffer. This system demonstrated safe encapsulation of neural stem cells (NSCs) in biological conditions without chemical initiators/UV light, achieving high cell viability. Cell viability in click gels was nearly double that of UV exposed gels after 1 d as well as 14 d of subsequent culture; demonstrating the sensitivity of NSCs to UV light damage, as well as the need to develop safer encapsulation strategies. Finally, protein immobilized click hydrogel *in vitro* differentiation over 2 wks demonstrated that the click gels specified primarily neurons without the need for additional protein differentiation factor media supplementation.

Keywords

click chemistry; hydrogel; neural stem cells; interferon- γ ; differentiation; neuron; immobilized protein

Introduction

Hydrogels are promising scaffolds for a variety of applications in regenerative medicine, mainly due to physical properties (e.g., stiffness, porosity, high water content, etc.) that are similar to native ‘mesenchymal’ tissues.¹ They offer tunable mechanical properties, high oxygen and nutrient permeability, as well as extracellular matrix (ECM)-like architecture to enhance cell growth and tissue formation.^{2–4} Hydrogels are especially applicable for recapitulating the ECM of the central nervous system (CNS), and have been specifically

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applied to neural stem/progenitor cell (NSPC) culture.^{5–8} The CNS can be described as a complicated structure that largely consists of cells surrounded by a soft crosslinked polysaccharide network integrating proteins and glycosaminoglycan side chains.^{9, 10} Cell-adhesive regions by order of prevalence in the CNS are laminin, fibronectin and collagen IV¹¹.

Hydrogels can be classified into physically and chemically crosslinked systems, based on the network formation mechanism utilized.^{12, 13} If properly designed with selective chemistry that limits unintentional interactions with cellular functional groups,¹⁴ a physically crosslinked hydrogel system will not require nonspecific chemical reactions for gelation and cells can safely be included/encapsulated. However, physical hydrogel systems typically have downsides, including difficulty offering a broad range of mechanical properties with control over the pore size and limited chemical functionality, which limit *in vivo* applications.^{15, 16} For a chemically crosslinked hydrogel, the bulk polymer or polymer solution network is covalently crosslinked. Chemically crosslinked hydrogels can be achieved by radical polymerization,^{17, 18} chemical reaction of complementary groups (mainly OH, COOH, NH₂), high energy irradiation, and enzymatic transformations.^{19, 20} In physically crosslinked hydrogels, polymer chains are held together by chain entanglements or physical interactions such as ionic interactions, hydrogen bonding or hydrophobic interactions.^{21, 22} Traditional chemically crosslinked hydrogel systems are prevalent in biomedical applications, however, many of the preferred techniques are problematic if cells are included during crosslinking because 1) crosslinking agents are often toxic to cells and leaching of the unreacted crosslinking chemicals can extend this damage,²³ 2) free radical generation during crosslinking reactions can induce cellular oxidative damage^{24, 25} and 3) ultraviolet (UV) crosslinking can cause direct protein and DNA damage in cells.²⁶ Despite these downsides, chemical crosslinking is often advantageous over physical crosslinking because it is easier to control and is more tunable. Thus, a highly efficient method that maximizes cell viability is needed for fabrication of chemical crosslinked hydrogels for safe and effective cell encapsulation.

Metal-free strain-promoted azide-alkyne “click” cycloaddition (SPAAC) has been extensively used for *in vivo* cell imaging,^{27, 28} three dimensional fibroblast cultures,²⁹ nanofibrous scaffold functionalization,^{30–32} and hydrogel fabrication.^{33–35} This reaction provides high yield with fast kinetics, occurs under mild reaction conditions (biological pH, room or body temperature and atmospheric pressure) and is biocompatible with cells and living animals.^{27, 29, 36} A previous study has shown that a copper-free click crosslinking hydrogel allows incorporation of adult human mesenchymal stem cells (hMSCs) with high viability maintained after 24 h.³³ However, the application of this SPAAC hydrogel with covalently tethered protein factors for other cell and tissue types, especially the nervous system, has not yet been tested. Importantly, the utility of tethering signaling proteins has recently been demonstrated to spatially control NSPC lineage commitment *in vivo* using a UV initiated crosslinked chitosan hydrogel system.³⁷ This study demonstrated that immobilization could maintain active growth factors *in vivo* to differentiate delivered NSPCs without the need for any resupplementation – as is typically needed when using soluble diffusing growth factors. This work built upon previous studies,³⁸ demonstrating that tethered interferon- γ (IFN- γ), a potent neurogenic factor, unexpectedly induced the

formation of neuroepithelium like structures over 4 weeks *in vivo*, a hallmark of early nervous system development. In this study, the primary goal was to implement a new clickable polymer platform into a hydrogel-based neural tissue engineering strategy and compare viability to UV exposed controls. To enable this, a covalently crosslinked hydrogel was created via reaction between dibenzocyclooctyne (DIBO)-functionalized polyethylene glycol (PEG) and four arm PEG tetraazide. The kinetics of hydrogel formation, equilibrium modulus and swelling ratio were characterized. Furthermore, covalently immobilized IFN- γ , laminin (an important protein found in the CNS known to enhance cell attachment, migration, differentiation and neurite extension^{39–42}), and encapsulated NSPCs were incorporated into this copper-free click hydrogel and its effect on viability and neuronal differentiation were investigated over 14 days as compared to controls with no IFN- γ and soluble IFN- γ that was resupplemented during media changes.

Materials and methods

Chemical synthesis, protein modification and hydrogel formation

Dibenzocyclooctynol (DIBO)-functionalized poly(ethylene glycol) (PEG) was synthesized using 4-nitrophenyl chloroformate activated 4-DIBO and poly(ethylene glycol) bis-amine (6 kDa), then purified as previously described.³³ Four arm PEG tetraazide (2 kDa) replaced the previously reported three arm PEG triazide crosslinker, and was purchased from Creative PEG Works (Winston Salem, NC, USA). The cytokine IFN- γ was recombinantly expressed and purified in house as described in previous studies.^{43, 44} To encourage cell attachment and to increase the local growth factor functional efficiency within the hydrogel, IFN- γ protein and laminin were reacted with azide-PEG4-NHS ester at a molar ratio of 1:20 for 3 h to add azide groups to the proteins to create azide-INF- γ and azide-laminin (Figure 1). This nonspecific form of modification targets primary amine containing amino acid residues.⁴⁵ For purification the protein reaction mixtures were placed in dialysis cartridges (10,000 MWCO; Thermo Scientific, Waltham, MA) and dialyzed against PBS for 3 d with 3 changes per day. Final stock protein concentrations were determined via μ BCA protein assay (Thermo Scientific). To form gels, 362 mg DIBO-functionalized PEG (0.06 mmol) was first dissolved in 1.81 mL ultrapure water. Azide-INF- γ and azide-laminin were added at concentrations of 300 ng/g and 100 ng/g, respectively, then allowed to react and couple. These concentrations were chosen to achieve final INF- γ and laminin concentrations of 150 ng/g and 50 ng/g, respectively, concentrations that have been shown to induce NSPC neuronal differentiation.⁴⁵ Next 60.33 mg (0.03 mmol) of PEG tetraazide (corresponding to an approximate 1:1 molar ratio of DIBO groups;azide groups upon combining with the DIBO-PEG solution) was dissolved in same amount ultrapure water. Finally the two solutions were mixed together at equal volume via pipetting and placed in a plastic well-plate to serve as the mold, within 5 min a hydrogel will begin to form.

Hydrogel Stiffness and Swelling Experiments

An oscillatory shear rheology experiment (ARES-G2, TA Instruments, New Castle, DE, USA) equipped with 8 mm parallel plates using a frequency of 10 rad/s (1.6 Hz) and a strain amplitude of 10% was performed to study gelation and kinetics. Measurements were obtained at 24 °C. DIBO-PEG based solution (described above) and PEG tetraazide (1:1

molar ratio) were added on top of the lower plate before measurement, which proceeded to equilibrium (~1000 s).

To estimate the swelling ratio, 100 μ L click gels (made as described above) were prepared and lyophilized. Dry mass (M_D) was measured, followed by swelling of dry hydrogel scaffolds in 1 X PBS at 37°C for 72 h. Hydrogels were carefully centrifuged on 50 μ m cell strainers (1500 RPM) to remove any PBS clinging to the edges of the scaffolds before each mass measurement. The mass after swelling (M_S) was determined when mass no longer changed and the swelling ratio (Q_M) was calculated with the following equation:

$$Q_M = \frac{M_S}{M_D}$$

NSPC harvest and cell culture

NSPCs were harvested from the lateral ventricles of the forebrain of adult rats (female Fischer 344 inbred rats, 8 weeks old) then expanded with growth medium consisting of neurobasal media (NBM), 2 mM L-glutamine, 100 μ g/mL penicillin-streptomycin, 2% (v/v) B27 (all Life Technologies, Carlsbad, CA, USA), 20 ng/mL epidermal growth factor (EGF-recombinant human; Sigma-Aldrich, St. Louis, MO, USA), 20 ng/mL basic fibroblast growth factor (bFGF; PeproTech, Rocky Hill, NJ, USA), 2 μ g/mL heparin (Sigma-Aldrich) in an incubator (37 °C, 5% CO₂), and cells counted and passaged weekly.^{39, 43, 46} For neuronal differentiation experimentation, passage six neurospheres were dissociated for cell seeding.

To make scaffolds, first proteins (azide-IFN- γ , IFN- γ , azide-laminin) were combined with the DIBO-PEG solution as described above except replacing water with cell growth medium. Then the cell suspension was added (5×10^6 cells/g) to achieve 20 wt% (DIBO-PEG). PEG tetraazide was prepared at 3.64 wt% in cell growth medium. 50 μ L of each solution was mixed via gentle pipetting and placed in a plastic well-plate to form cell seeded hydrogels. To study and compare how UV light exposure and a free radical initiator could affect cell viability, four treatment groups were selected. These consisted of the hydrogel containing 1. azide-IFN- γ (immobilized) or 2. IFN- γ (soluble control). The hydrogel containing 3. azide-IFN- γ that was subsequently UV irradiated (365 nm, 2.7 mW/cm²) after gelation for 3 min. The final group, was 4. the hydrogel containing azide-IFN- γ and 3 μ L/g (final concentration in hydrogel) photoinitiator 1-hydroxycyclohexyl phenyl ketone in 1-vinyl-2-pyrrolidinone (stock at 300 mg/mL; Sigma-Aldrich) irradiated (365 nm, 2.7 mW/cm²) for 3 min. All scaffolds were next cultured in NSPC growth medium without EGF, FGF or heparin. 150 ng/mL IFN- γ was included in the growth media for the soluble control group only. Medium changes were performed at day 1 and day 7. 9 scaffolds were made for each group to perform the analyses described below.

LIVE/DEAD viability testing

At day 1 and 14, 3 scaffolds were selected from each group, were cut in half, then analyzed using the LIVE/DEAD Cell Viability Assay following the manufacture's protocol (Life Technologies), then imaged on an inverted fluorescent microscope (Olympus IX-81, Tokyo,

Japan). An additional two control groups containing NSPCs floating in growth media alone (one treated with 3 min UV light, the other serving as a no treatment control) were also analyzed by Live/Dead after 1 d. Viability of NSPCs in click gels were analyzed at day 1 and 14 with the primary aim of determining click hydrogel cell viability while comparing the effects of UV light exposure.

Immunohistochemistry

At day 14, three scaffolds from each group (immobilized, soluble and IFN- γ control) were fixed with cold methanol (-20°C) for 10 min in the freezer (-20°C), then washed with PBS for cryosectioning then immunohistochemistry (IHC). The following primary antibodies and dilutions were used for IHC staining: monoclonal mouse anti- β -III tubulin (1:1000, Covance, Princeton, NJ, USA) identified neurons; monoclonal anti-RIP (1:5, Developmental Studies Hybridoma Bank, Iowa City, IA, USA) identified oligodendrocytes; monoclonal mouse anti-glial fibrillary acidic protein (GFAP) (1:400, Millipore, Billerica, MA, USA) identified astrocytes; and monoclonal mouse anti-nestin (1:20, Developmental Studies Hybridoma Bank) were used for staining NSPCs. Secondary goat anti-mouse IgG Alexa-Fluor 546 (1:400, Life Technologies) were used to target primary antibodies. Mounting was achieved with ProLong Gold antifade reagent (Life Technologies). Multiple sections and slides were fluorescently imaged (Olympus IX-81) for each group and positive cells were quantified as has been described previously.³⁹

Statistics

All statistical analyses were performed using JMP 10 (SAS Institute, Cary NC, USA). ANOVA with Tukey's post-hoc analysis was performed to detect significant differences between groups. An α level of 0.05 was used to determine significance between groups. Data are reported as mean $\pm$ standard deviation (SD).

Results and discussion

Results from rheology and swelling ratio testing are presented in Figure 2. At the beginning of the rheology experiment, the solution was a viscous liquid. G' and G'' values increased along with reaction time as the materials crosslinked and transformed from a viscous liquid to a viscoelastic solid. Rheology showed the dynamic modulus arrived at equilibrium values after 1000 s (Figure 2A), indicating the crosslinking was nearly complete. The equilibrium click gel complex shear modulus determined from 3 independent samples was 0.703 ± 0.002 kPa, which is in the optimal range of CNS hydrogel modulus.⁴⁷ Compared to a previous study that used a three armed PEG triazide crosslinker (1.1 kDa), rheological properties are similar and gelation equilibrium was reached in $\sim 1/6$ the time.³³ Comparable gelation times were observed as reported in a recent study that employed a similar four arm DIBO/azide crosslinked PEG system.⁴⁸ The inclusion of tethered proteins likely had little effect on rheological and swelling properties since the molar concentrations of azide-laminin and azide-INF- γ that reacted with DIBO-PEG were quite small ($\sim 10^{-13}$ mol/g), which is well below the molar concentration of DIBO-PEG that was used ($\sim 10^{-5}$ mol/g).

The swelling revealed that equilibrium was reached after incubation in PBS for 1 d and Q_M was 24.02 ± 2.91 (Figure 2B). From the swelling results, a relatively quick swelling process was observed during the first couple of hours, with equilibrium reached by 24 h. Similar swelling ratio results have been shown in other click gel systems.⁴⁹ The high swelling ratio of the hydrogels presented in this study and quick swelling process suggest high porosity with interconnected open-cell structure, which are advantageous for tissue engineering strategies.⁵⁰

Viability of NSPCs in click gels were analyzed at day 1 and 14 (Figures 3,4). At day 1, quantification of viable cells revealed that the percentage of viable cells in click gels was similar for immobilized IFN- γ and soluble IFN- γ groups ($86.89 \pm 6.15\%$, $82.78 \pm 9.52\%$). At day 1 soluble IFN- γ + UV ($46.30 \pm 5.32\%$) and soluble IFN- γ + UV + initiator ($49.20 \pm 0.80\%$) significantly decreased cell viability as compared with no UV treatment groups ($p < 0.0001$). At day 14, the number of viable cells without UV treatment still remained between 70% and 75% (Figure 4) and was still significantly larger compared with UV treated groups (44% – 47%; $p < 0.0001$). Similar results were also observed for the growth media control groups (no treatment day 1 $72.34 \pm 7.53\%$, UV 3 min exposure $46.30 \pm 5.32\%$). In previous studies of cell viability in UV-radical initiated hydrogels, chemical initiator concentration and UV exposure have been shown to also influence cell viability (human fetal osteoblasts, bovine chondrocytes, corneal epithelial cells, human mesenchymal stem cells, goat mesenchymal stem cells, human embryonic germ cells and fibroblasts),^{51, 52} and were proven to lead to significant cell viability decreases during 3D cell encapsulated hydrogel culture.⁵³ Interestingly, recent work has shown that the choice of polymer reactive moiety directly impacts bioactivity following UV initiated polymerization, and acrylates offer no protection and are very poor compared to thiol-enes.⁵⁴ In this study, day 1 cell viability was 80% – 90%, with this cell loss likely caused from the cell passaging process (mechanical dissociation), at day 14 cell viability slightly decreased (75% – 80%), but was not significant compared with day 1 (Figure 4).

Finally, cell differentiation studies were performed and analyzed using IHC staining of fixed and cryosectioned scaffolds. The primary goal for these studies was to demonstrate that immobilized IFN- γ results in the same neuronal differentiation as soluble IFN- γ (maintained at the same effective concentration), similar to what has been previously shown in chitosan-based hydrogel systems.^{43, 55, 56} Furthermore, these previous studies have demonstrated that tagged and purified proteins (especially IFN- γ) can be very efficiently immobilized – nearly 100% based on the initial amount of tagged protein added for conjugation. Quantification of NSPC differentiation in click gel scaffolds (immobilized IFN- γ , soluble IFN- γ and no IFN- γ control) was performed after 14 d (Figure 5, Supplementary Figure 1). From the results, significantly more β III tubulin positive neurons were observed compared with other cell types (oligodendrocytes (RIP+), astrocytes (GFAP+), uncommitted NSPCs (nestin+)), as has been reported previously in other encapsulated hydrogel systems.^{38, 39} As desired, neuronal differentiation by IFN- γ treatment showed that the immobilized group resulted in similar neuronal differentiation percentages (β -III tubulin positive cells) compared with soluble groups ($63.33 \pm 4.64\%$ vs. $53.33 \pm 8.47\%$; $p > 0.05$). There was no significant difference observed between the IFN- γ hydrogel groups for oligodendrocyte and astrocyte differentiation by RIP and GFAP staining ($p > 0.05$). Nestin staining for NSPCs

showed small percentages of positive cells and no significant difference between immobilized and soluble groups ($p > 0.05$). The control group lacking IFN- γ (immobilized or soluble) was 100% nestin positive with no other positive staining, indicating no differentiation during the culture period while confirming the action of both soluble and most importantly immobilized IFN- γ . Similar neuronal differentiation percentages (~ 60 to 70%) have been reported in previous studies using chitosan-based hydrogels with IFN- γ ^{43, 55, 56}. Similar control experiments were also included showing 100% nestin positive cells after 14 d of culture, as was observed in this study.³⁹ It is important to note that the cells in the staining images still mostly appear rounded in shape within the hydrogel, and this may be due to network entrapment and the simple fact that cell-matrix and cell-cell interactions are not maximized in these MAC scaffolds. In the future it would be valuable to investigate improving these aspects (e.g., including enzyme cleavable crosslinks⁵⁷) and thus the potential regenerative utility of the system.

Conclusions

NSPCs offer an attractive means of regenerating lost or damaged CNS tissue after disease or injury and research has shown that hydrogels are most appropriate as inductive scaffolds since NSPCs, neurons and mature glia prefer very soft substrates.⁴⁷ Neuronal differentiation is the priority, since upon implantation to a CNS injury, reestablishment of neuronal connections are requisite for recovery of CNS function. Several hydrogel systems for neural tissue engineering have been described previously, including but not limited to other PEG,⁵⁸ agarose,⁵⁹ collagen⁶⁰ and chitosan-based hydrogels.³⁹ To date, immobilized lineage commitment factors are uncommon and all of these reported encapsulation approaches are partially cytotoxic to NSPCs, either requiring radical generation, UV light with unprotected reactive groups, or change of temperature or pH. NSPCs are especially sensitive to these and other environmental factors and should be considered in all biomaterial delivery strategies.⁶¹

In summary, this work illustrates the utility that the copper-free azide-alkyne click reaction offers for encapsulating neural stem cells in hydrogels for potential future regenerative medicine applications. Herein, this hydrogel system maintains high cell viability after encapsulation. Further, the system offers the ability to easily include immobilized attachment and signaling proteins to efficiently direct NSPC differentiation to neurons without the need for additional supplementation. The primary advantage of this click-gel system is the ability to offer a non-biological, gentle crosslinking reaction to reduce cell damage and death during hydrogel formation. Additionally, the bio-orthogonal nature and appropriate mechanical properties of this click hydrogel provides great potential in neural regeneration applications where the delivery of stem cells and treatment molecules could be advantageous for treating acute or chronic neurodegenerative conditions, including but not limited to Parkinson's, traumatic brain and spinal cord injuries, and stroke.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Schematic for neurogenic click hydrogel network formation. (A) DIBO-PEG reacted with azide-IFN- γ or separately azide-laminin to form DIBO-PEG-proteins for subsequent reactions. (B) DIBO-PEG-protein, PEG tetraazide plus NSPCs form a stem cell encapsulated hydrogel able to specify neuronal differentiation in 3D.

Figure 2.
 (A) Modulus-time dependence during crosslinking via oscillation shear rheology testing (24 °C), representative curve from 3 independent samples. (B) Click gel swelling results in PBS at 37 °C. $n=3$, mean $\pm$ SD.

Figure 3. Live/Dead assay results indicate cell viability differences when UV light exposure occurred (3 min, 2.7 mW/cm²) at day 1 and day 14. Live cells stain green, dead cells stain red.

Figure 4.

Cell viability assayed by Live/Dead tested at day 1 and day 14 for NSPCs exposed to the listed treatments. *** denotes significance by two-factor ANOVA ($p < 0.0001$), day 1 vs 14 was not significantly different ($p > 0.05$). $n=3$, mean $\pm$ SD.

Figure 5.

Fluorescence staining results for NSPC differentiation. (A) Quantification of IHC at day 14 shows similar neuronal percentages ($p > 0.05$) for both immobilized and soluble groups. Nestin was significantly higher in the control treatment (no IFN- γ ; $p < 0.0001$); $n=3$, mean $\pm$ SD. (B) Representative photomicrographs of fluorescence staining of scaffold cross-sections for neurons. Nuclei appear blue by Hoechst 33342, positive cell staining for each differentiation marker appear red by Alexa Fluor 546.