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A tunable synthetic hydrogel system for culture of retinal ganglion cells and amacrine cells

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Abstract

The central nervous system (CNS) consists of complex groups of individual cells that receive electrical, chemical, and physical signals from their local environment. Standard *in vitro* cell culture methods rely on two-dimensional (2D) substrates that poorly simulate *in vivo* neural architecture. Neural cells grown in three-dimensional (3D) culture systems may provide an opportunity to study more accurate representations of the *in vivo* environment than 2D cultures. Furthermore, each specific type of neuron depends on discrete compositions and physical properties of their local environment. Previously, we developed a library of hydrogels composed of poly(ethylene glycol) (PEG) and poly(L-lysine) (PLL) which exhibit a wide range of mechanical properties. Here, we identified specific scaffolds from this library that readily support the survival, migration and neurite outgrowth of purified retinal ganglion cells (RGCs) and amacrine cells (ACs). These data address important biological questions about the interaction of neurons with the physical and chemical properties of their local environment and provide further insight for engineering neural tissue for cell-replacement therapies following injury.

Keywords

ECM (extracellular matrix); Protein adsorption; Elasticity; Retina

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INTRODUCTION

Two-dimensional (2D) culture systems provide a practical solution for studying survival and neurite outgrowth of neurons in culture, however three-dimensional (3D) culture systems can potentially deliver higher degrees of control and organization of cellular environments, more physiologically relevant cell migration, and support the formation of more extensive 3D networks of cell-cell interactions[1, 2]. Furthermore, cells alone are often not capable of recreating complex tissues on their own although stem and progenitor cells may form 3D tissues under certain conditions[3]. Nevertheless, on a 3D scaffold, cells may recapitulate features of complex tissues not otherwise observed in 2D cultures. Hydrogels are excellent candidates for 3D scaffolds for *ex vivo* culture of mammalian cells. Hydrogels demonstrate mechanical properties that parallel the properties of soft tissues[4]. The hydrogel's aqueous environment protects cells, allows for transport of nutrients and metabolite exchange, permits physical, chemical, and biological modification, and demonstrates a generally high biocompatibility [5].

In our previous work we developed a library of hydrogels based on poly(ethylene glycol) (PEG) and poly(L-lysine) (PLL) which exhibited a wide range of mechanical properties, a subset of which promoted neural differentiation of neural progenitor cells [6]. Our library of PEG/PLL hydrogels exhibits a wide range of mechanical properties that can be varied independently of the charged PLL component to investigate the role of mechanical modulus on cellular behavior including migration, organization, and differentiation [7]. To ask if these scaffolds can support survival and neurite growth of mature neurons, we screened the library of hydrogels, beginning with those identified as good substrates for promoting NSC differentiation. In the appropriate hydrogel environment, we found that acutely purified retinal ganglion cells (RGCs) and amacrine cells (ACs) readily survive and extend extensive neurite outgrowth following seeding onto PEG/PLL-based hydrogel scaffolds.

MATERIALS AND METHODS

Materials

Four-arm poly(ethylene glycol) (PEG Mn 2000 g/mol and 10,000 g/mol) was obtained from Nektar Therapeutics (Huntsville, AL). Poly(L-lysine) hydrobromide (PLL MW 70–150 kDa and 150–300 kDa) and all other reagents were obtained from Sigma (St. Louis, MO) and used as received. SpectraPor dialysis membranes (MWCO 1000 Da and 6000–8000 Da) were obtained from Spectrum Laboratories (Rancho Dominguez, CA) and rinsed thoroughly with deionized water before use.

Synthesis of Activated PEG

Carbamate cross-links between PEG and PLL macromers (Fig. 1A) were created through N,N-carbonyldiimidazole (CDI) activation of PEG as described previously[6, 8]. Briefly, PEG was dissolved in an excess of dioxane at 37°C. For every hydroxyl present on PEG, a 1:8 molar excess of CDI was added. The resulting mixture was stirred under argon for 2 h at 37°C. Unreacted CDI was removed by dialysis in deionized water for 48 h. The resulting solution was flash frozen in liquid nitrogen and lyophilized for 3 days. Activated PEG was stored in a desiccator at –20°C.

Fabrication of Hydrogels

All hydrogels were fabricated as 10% w/v polymer in 1X phosphate buffer solution (PBS). A range of PLL and activated PEG ratios were dissolved in 1X PBS, mixed vigorously, and allowed cure/cross-link at 25°C for 24–36 h. The PLL and activated PEG ratios chosen were based on a subset of gels from the library of gels previously created[6]. These particular gels

were chosen for two reasons: (1) their ability to polymerize without additional chemical modification, and (2) their ability to influence neural stem cell survival and differentiation[6].

Hydrogel Morphology

To ensure the swollen architecture of the hydrogel would be maintained for imaging, a multi-step dehydration process was used. Gels were swollen in 1X PBS for 12-18 h, frozen overnight at -20°C , placed in a liquid nitrogen bath for 1 h, and then lyophilized for 24 h. Gel cross-section samples were mounted and sputter coated with gold for 30 s at 40 mA. The morphology of the hydrogels were then evaluated by scanning electron microscopy (SEM, Philips XL-30 environmental SEM operating at 10 kV).

Rheology

Elastic moduli for swollen hydrogels were determined as previously described [6]. Briefly, hydrogels were cured and then swollen overnight in 1X PBS. 20-mm discs were then cut from the swollen hydrogel and tested. Elastic moduli were obtained using a Stress Control Rotational Shear Rheometer-AR1000 (TA Instruments, New Castle, DE) with acrylic 20-mm diameter parallel plate geometry. Moduli were measured with a constant normal force of 2 N.

Preparation of Hydrogels for Cell Studies

Hydrogels were swollen in DPBS for 18 h to allow for equilibrium swelling. Following swelling, uncoated gels (1 mm $\times$ 5 mm) were sterilized by UV exposure (30 min, 5 mW/cm²). The UV light source was two feet away from the scaffold plates. To enhance attachment of RGCs and ACs, the gels were coated with sterile laminin (2 mg/mL; Telios/Invitrogen) for at least 24 hours, allowing neutralization of charge effects of any free amines. Gels were thoroughly rinsed with sterile DPBS prior to cell seeding (Fig. 1B).

RGC and AC Purification and Culture

RGCs and ACs were purified by immunopanning as previously described [9, 10]. Briefly, postnatal rat retinas were dissociated with papain (Worthington) and mechanically triturated to obtain a single-cell suspension. The retinal suspensions were first depleted of rat macrophages by immunopanning (1:75, AI A51240; Accurate Chemical, Westbury, NY). Using T11d7 (Thy-1) coated dishes, RGCs were purified to over 99% purity as confirmed by RGC specific markers (data not shown). Enrichment of ACs to 88% purity was achieved after depleting rat macrophages and T11d7- and Ox7-positive cells and immunopanning for VC1.1-positive cells [11]. Cells were released from cell culture dishes with a combination of trypsin (0.02% in DPBS) and mechanical trituration. RGCs and ACs were seeded at 50,000 cells per hydrogel or on 24 well plates coated with poly-D-lysine (PDL) (70 kDa, 10 $\mu\text{g}/\text{mL}$; Sigma) and laminin (2 mg/mL; Telios/Invitrogen) in serum-free defined medium containing BDNF (50 ng/mL; Peprotech), CNTF (10 ng/mL; Peprotech), insulin (5 $\mu\text{g}/\text{mL}$; Sigma), and forskolin (5 μM)[9, 10].

RGC and AC Survival Assay

After four days in culture, growth media was replaced with neural growth media containing 1 μM calcein-AM (Invitrogen, Carlsbad, CA) and 4 μM propidium iodide (Invitrogen) for 5 minutes in a 37°C 10% CO₂ incubator. The culture media was then removed and replaced with fresh neural cell growth media. Live cells were immediately imaged under confocal microscopy (Leica TCS SP5 Confocal Microscope). Using a 10x dipping objective, 50- μm Z-stack projections were acquired under fluorescence confocal microscopy. Three visual fields were randomly sampled from each scaffold; at least 100 cells were counted from each

field, and three scaffold replicates were analyzed for each condition. Live (calcein-positive, PI-negative) and dead (PI-positive) cells were manually counted.

RGC Neurite Direction and Orientation Assay

The orientation of neurites of RGCs on scaffolds were measured using the ImageJ plugin OrientationJ, written by Daniel Sage and available at <http://bigwww.epfl.ch/demo/orientation/>. The ellipses were made using the 'measure' function of the plugin with the Laplacian of Gaussian set to zero. The eccentricity of the ellipses demonstrates calculated coherence of the direction of neurites. The narrower the ellipse the higher the coherence, and the direction of the ellipse also indicates the direction of lines in the region of interest measured. A perfect circle would denote a completely random configuration. The frequency of neurites extending toward an arbitrary direction was measured by the Distribution function of the OrientationJ plugin. The Gaussian Gradient was used with a sigma value of 1, a minimum coherence of zero, and a minimum energy of 0. Values from the output were normalized and then wrapped around 90° for each region to indicate the direction toward the 90° vertical. Data were partitioned into three bins of 30° each. The Shannon entropy of the θ distribution from the histograms are calculated as $H_\theta = -\sum p_n \ln(p_n)$, where p_n is the probability of a given orientation. This value quantifies the uncertainty of the θ distribution and measures the extent to which neurite growth is oriented to a particular angle, where the greater the entropy, the higher amount of randomness.

RESULTS

Hydrogel Morphology and Elasticity

Selected hydrogels (Table 1) from the library were synthesized and their morphology was characterized. SEM of the swollen synthesized hydrogels revealed an open, porous network with pores on the order of 20-200 μm (Fig. 2). Elastic moduli of the hydrogels, as determined by rheology in the previous study[6], varied from 1406 ± 139 Pa for hydrogel D1b to 23146 ± 1547 Pa for hydrogel D4c (Table 2).

RGCs and ACs Survive and Extend Elaborate Neurites on PEG/PLL Hydrogels

We first asked whether primary RGCs and/or ACs could survive following transplantation onto hydrogel scaffolds. A library of scaffolds was generated to screen for physical and chemical properties which best supports RGC and AC survival. Postnatal RGCs and ACs were purified to homogeneity by immunopanning, and pipetted directly on the surface of each specific scaffold type. We observed robust survival of both RGCs and ACs on specific types of scaffolds (Fig. 3B), while other scaffolds failed entirely to support neuronal survival (Fig. 3A). Closer examination of these data revealed a preference for specific chemical characteristics of the hydrogels. RGCs and ACs had better survival, 50% or greater, on hydrogels composed of high molecular weight PEG and a ratio of 3:1 or 4:1 amines to hydroxyls (Fig. 3C and Table 2). These hydrogels that demonstrated superior cell survival had a range in elastic moduli, from approximately 3800 Pa to 5800 Pa. No correlation between survival and moduli was found. The cell morphology of RGCs or amacrine cells did not differ among the hydrogels that supported their survival. Thus RGCs and amacrine cells require specific hydrogel properties to promote neuronal survival.

We next asked if surviving RGCs and ACs could extend neurites on the hydrogel scaffolds. Using confocal microscopy, we observed that purified RGCs and ACs were capable of extending lengthy neurites, often greater than 500 μm in length. RGCs generally displayed dendritic-like arborization around the soma and one long axon containing a growth cone, similarly to what is observed both *in vivo* and *in vitro* on 2D, PDL/laminin-coated dishes (Fig. 4C). ACs displayed dendritic-like arborization with star-like morphologies similarly to

what is observed *in vivo* and *in vitro* on PDL/Laminin-coated dishes (Fig. 3B). When we measured how many of the cells were capable of extending at least one neurite over 200 μm , we found that the scaffolds that supported the survival of both RGCs and ACs were readily capable of supporting lengthy neurite outgrowth (Fig. 4D). Using confocal Z-stack projections, we observed that most of the neurites of both RGCs (Fig. 4C) and ACs were found on the surface of the scaffolds (Fig. 4C top image), but some projections were found to penetrate deeper into the scaffold structure (Fig. 4C bottom image). Thus, purified primary RGCs and ACs extend lengthy neurites on hydrogel scaffolds.

We next asked if any of the hydrogel scaffolds affect the direction and orientation of the neurite outgrowth of RGCs. The orientation of neurite outgrowth of RGCs cultured on hydrogel scaffolds (Fig. 5A) were measured using the ImageJ plugin OrientationJ. The frequency of neurites extending toward an arbitrary direction was measured by the distribution function of the OrientationJ plugin. When we partitioned the data into three bins of 30° , we found that for all scaffolds, the percentage of neurites going in a specific direction ($0\text{-}30^\circ$) was not significantly different ($p < 0.01$) than those away from it ($60\text{-}90^\circ$) or in neutral directions ($30\text{-}60^\circ$) (Fig. 5B). Randomness was measured in terms of entropy (Fig. 5C). All the scaffolds that supported neurite had a similar level of randomness associated with the direction of neurite outgrowth. This demonstrates that although the hydrogel scaffolds support the elaborate growth of neurites, the direction of the neurites is random. This suggests that none of the scaffolds tested provide direction or orientation cues to direct neurite outgrowth towards a particular direction.

DISCUSSION

Here we characterize the survival and neurite growth of two purified primary retinal neurons. The retina, as a developmental outgrowth from the brain, is part of the central nervous system, but is an excellent model for bioengineering approaches because it is patterned into layers of specific cells *in vivo*. RGCs and ACs are born during embryonic development from a common retinal progenitor. These two cell types synaptically integrate with one another in the same retinal plexiform layer. As previously reported by our lab, most genes ($>74\%$) are expressed at similar levels in RGCs and ACs at embryonic and postnatal ages suggesting that they may share some overlapping signals regulating survival, neurite outgrowth, migration and other properties required for integrating into an environment [11]. Here we add to this characterization, finding that similar types of scaffolds supported survival and neurite outgrowth for both RGCs and ACs. Thus these data reflect a unique opportunity to directly compare two purified primary neurons that normally interact *in vivo* across different layers of the retina, to examine whether there are any differences in their response to bioengineered substrates.

Of course, there are critical differences between these scaffolds and the *in vivo* retinal environment. *In vivo*, the extracellular matrix (ECM) is comprised of a complex organization of glycoproteins, collagens, and hyaluronic acid (HA), among other components. The architectural scaffolding provided by the ECM, in addition to the proteins sequestered in the matrix, influence a cell's ability to differentiate, proliferate, and migrate [12-14], and in the CNS also regulates neuronal survival, axon growth, and synapse formation [15, 16]. In order to understand cell behavior within complex tissues like the retina or the rest of the CNS, cells must be systematically studied in the context of a model microenvironment, such as the model proposed here. Other models, including explanting the whole retina, studying retinal slices, or studying cells in culture dishes, provide little opportunity to simplify the environment while maintaining a 3D cellular milieu. The system presented here provides a new, customizable research tool to study cells in a 3D environment. Such studies could influence the application of scaffold and polymer science

to the in vivo environment, as for example in situ injection of dipeptide-based polymers may enhance RGC survival after optic nerve injury [17].

This contrasts with the utility of non-customizable or non-tunable polymer scaffolds such as Matrigel™, a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm mouse sarcoma primarily composed of laminin, collagen type IV, heparan sulfate proteoglycans, entactin, and various growth factors[18, 19]. While many investigators use Matrigel™ to study various cell types, the cocktail of ECM proteins and growth factors may not always be appropriate for the intended research. Use of a synthetic polymer as the base for the 3D matrix provides an opportunity to tailor the mechanical and chemical properties of the scaffold to suit the intended research purposes.

We previously reported reproducible synthesis of a series of PEG/PLL hydrogels with a range of chemical and mechanical properties. PEG is a highly biocompatible polymer that adds mechanical integrity to the hydrogel system. However, cells do not adhere to PEG, therefore incorporation of ECM-binding proteins into the network is required. To simplify our system we synthesized the PEG hydrogels with PLL, which non-specifically promotes cell adhesion[20]. In addition, since PLL contains free amines, the hydrogel can be further modified for specific research purposes.

While the RGCs and ACs responded favorably to half of the hydrogels we tested, survival on these hydrogels varied between the cell types. Hydrogels with a 3:1 or 4:1 ratio of amines to hydroxyls and high molecular weight PEG, regardless of PLL molecular weight, provided the best substrate for the RGCs to attach to. None of the hydrogels that had higher ratios of amines to hydroxyls demonstrated good survival for either cell type. This is consistent with observations that polycationic polymers with high free amine concentrations can be cytotoxic[21]. Furthermore, hydrogels with low free amine concentrations displayed decreased survival as well.

The mechanical properties, especially the stiffness, of the scaffold can play a large role in influencing the behavior of the cells. Scanning force microscopy-based experiments determined the elastic modulus K of the retinal inner surface along the length of the eye with sub-millimetre resolution. The inner retina is a mechanically inhomogeneous tissue with most elastic moduli in the range of 940 to 1800 Pa [22]. In the case of the hydrogels in our library, the RGCs survived best on hydrogels with elastic moduli ranging from 3800-5700 Pa; it is not known why RGCs prefer scaffolds in this higher range once explanted ex vivo. However, there were some hydrogels within this range of moduli where RGCs demonstrated poor survival. This demonstrates the importance of not only the stiffness of the hydrogels in influencing cell RGC and AC behavior, but the importance of the chemical composition as well. Interestingly, in our previous work with NSCs, hydrogels which were not favorable for NSC attachment and survival could be made favorable by coating the hydrogels with laminin[6], but this was not true for experiments with RGCs and ACs here. This may be because laminin was not as effective at neutralizing free amines, unlikely from the prior measurements [6]. We hypothesize that the more likely explanation is that RGCs may secrete less ECM and ECM-modifying enzymes such as matrix metalloproteinases (MMPs) compared to NSCs. RGCs are not the major source of MMPs and do not produce much ECM after optic nerve or retinal injury in vivo [23, 24]. This may also explain the relatively greater ability of NSCs to remodel the scaffolds and penetrate more deeply than the differentiated primary neurons studied here.

In the normal eye, RGCs exhibit highly polarized morphology with intra-retinal axons directed down well-aligned tracts towards the optic nerve head [25]. Therefore there is great interest in recapitulating these precise levels of organization on neural-scaffolds engineered

for retinal repair [26, 27], and recent work using electrospun scaffolds has demonstrated a capacity to orient RGC axons to mimic retinal architecture [28]. Our data suggest that the hydrogel scaffolds tested in this experiment would require further alterations in order to introduce direction selective cues. Although the direction of neurite outgrowth was entirely random, both cell types survived and extended neurites on the same type of scaffolds, and each cell type still retained features of their stereotyped morphology including longer axons on RGCs and shorter neurites branching out of amacrine cells, suggesting that the scaffold environment was not sufficient to alter key morphological properties intrinsic to each cell type [11]. Of course the neurons did not retain other morphologic features normally seen *in vivo* when cultured on the scaffold environment. For example, RGC axon growth was not aligned nor fasciculated into axon bundles, as would normally happen in the nerve fiber layer *in vivo*.

Using this model system, we have begun to address a number of important biological questions about neuronal integration and can begin to ask which signals participate or influence these processes, as well as to take more dramatic steps towards an organ-replacement strategy for neural tissue. The impact of creating 3D neural tissue model systems will enhance studies not only of all aspects of retinal function, but also of the development and function of the brain. Future studies may incorporate neurons derived from patients with specific diseases, using retinal stem cells for example, to enhance our ability to study how 3D tissue architecture responds differently to insults or therapies. Furthermore, we propose that a well-characterized 3D tissue-engineered retina will provide many advantages in cost, animal usage, and assay specificity for programs interested in gene or drug screening. Finally, it will be a major step forward in cell replacement therapies currently being studied, to develop a 3D neural tissue platform as a starting point to study tissue/organ replacement therapy for the eye or brain, enhancing our understanding of the basic mechanisms of neuronal integration in these approaches, rebuilding neural circuitry *in vitro*, and replacing whole circuits *in vivo* [29].

CONCLUSIONS

Cell delivery systems, such as hydrogel scaffolds, offer much promise for enhancing cell-based therapies for glaucoma and other neurodegenerative diseases. Here, we identified specific scaffolds from a library that readily support the survival, migration and neurite outgrowth of purified retinal ganglion cells (RGCs) and amacrine cells (ACs). We found that RGCs and amacrine preferred scaffolds containing a range of physical and chemical properties with an elastic moduli range of 3800-5700 kPa. These data enhance our ability to mimic the properties found in specific lamina of the retina to generate 3D neural tissue platforms to better understand neuronal integration and transplantation.

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REFERENCES

- [1]. Griffith LG, Swartz MA. Capturing complex 3D tissue physiology in vitro. *Nat Rev Mol Cell Biol.* 2006; 7:211–24. [PubMed: 16496023]

- [2]. Lutolf M, Hubbell J. Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. *Nat Biotechnol.* 2005; 23:47–55. [PubMed: 15637621]
- [3]. Eiraku M, Takata N, Ishibashi H, Kawada M, Sakakura E, Okuda S, et al. Self-organizing optic-cup morphogenesis in three-dimensional culture. *Nature.* 2011; 472:51–6. [PubMed: 21475194]
- [4]. Tibbitt MW, Anseth KS. Hydrogels as extracellular matrix mimics for 3D cell culture. *Biotechnol Bioeng.* 2009; 103:655–63. [PubMed: 19472329]
- [5]. Hoffman AS. Hydrogels for biomedical applications. *Annals of the New York Academy of Sciences.* 2001; 944:62–73. [PubMed: 11797696]
- [6]. Hynes SR, Rauch MF, Bertram JP, Lavik EB. A library of tunable poly(ethylene glycol)/poly(L-lysine) hydrogels to investigate the material cues that influence neural stem cell differentiation. *J Biomed Mater Res A.* 2008
- [7]. Ford MC, Bertram JP, Hynes SR, Michaud M, Li Q, Young M, et al. A macroporous hydrogel for the coculture of neural progenitor and endothelial cells to form functional vascular networks in vivo. *Proc Natl Acad Sci U S A.* 2006; 103:2512–7. [PubMed: 16473951]
- [8]. Ford M, Bertram J, Hynes S, Michaud M, Li Q, Young M, et al. A macroporous hydrogel for the coculture of neural progenitor and endothelial cells to form functional vascular networks in vivo. *Proc Natl Acad Sci USA.* 2006; 103:2512–7. [PubMed: 16473951]
- [9]. Barres BA, Silverstein BE, Corey DP, Chun LL. Immunological, morphological, and electrophysiological variation among retinal ganglion cells purified by panning. *Neuron.* 1988; 1:791–803. [PubMed: 2908449]
- [10]. Meyer-Franke A, Kaplan MR, Pfrieder FW, Barres BA. Characterization of the signaling interactions that promote the survival and growth of developing retinal ganglion cells in culture. *Neuron.* 1995; 15:805–19. [PubMed: 7576630]
- [11]. Kunzevitzky NJ, Almeida MV, Goldberg JL. Amacrine cell gene expression and survival signaling: differences from neighboring retinal ganglion cells. *Invest Ophthalmol Vis Sci.* 2010; 51:3800–12. [PubMed: 20445109]
- [12]. Friedl P. Proliferation and plasticity: shifting mechanisms of cell migration. *Curr Opin Cell Biol.* 2004; 16:14–23. [PubMed: 15037300]
- [13]. Lin CQ, Bissell MJ. Multi-faceted regulation of cell differentiation by extracellular matrix. *FASEB J.* 1993; 7:737–43. [PubMed: 8330681]
- [14]. Nelson C, Bissell M. Of extracellular matrix, scaffolds, and signaling: tissue architecture regulates development, homeostasis, and cancer. *Annu Rev Cell Dev Biol.* 2006; 22:287–309. [PubMed: 16824016]
- [15]. Venstrom KA, Reichardt LF. Extracellular matrix. 2: Role of extracellular matrix molecules and their receptors in the nervous system. *FASEB J.* 1993; 7:996–1003. [PubMed: 8370483]
- [16]. Viapiano MS, Matthews RT. From barriers to bridges: chondroitin sulfate proteoglycans in neuropathology. *Trends Mol Med.* 2006; 12:488–96. [PubMed: 16962376]
- [17]. Nakatani M, Shinohara Y, Takii M, Mori H, Asai N, Nishimura S, et al. Periocular injection of in situ hydrogels containing Leu-Ile, an inducer for neurotrophic factors, promotes retinal ganglion cell survival after optic nerve injury. *Experimental eye research.* 2011; 93:873–9. [PubMed: 22001716]
- [18]. Kleinman HK, McGarvey ML, Hassell JR, Star VL, Cannon FB, Laurie GW, et al. Basement membrane complexes with biological activity. *Biochemistry.* 1986; 25:312–8. [PubMed: 2937447]
- [19]. Kleinman HK, McGarvey ML, Liotta LA, Robey PG, Tryggvason K, Martin GR. Isolation and characterization of type IV procollagen, laminin, and heparan sulfate proteoglycan from the EHS sarcoma. *Biochemistry.* 1982; 21:6188–93. [PubMed: 6217835]
- [20]. Rainaldi G, Calcabrini A, Santini MT. Positively charged polymer polylysine-induced cell adhesion molecule redistribution in K562 cells. *Journal of materials science Materials in medicine.* 1998; 9:755–60. [PubMed: 15348935]
- [21]. Wang Y-X, Robertson JL, Spillman WB, Claus RO. Effects of the chemical structure and the surface properties of polymeric biomaterials on their biocompatibility. *Pharm Res.* 2004; 21:1362–73. [PubMed: 15359570]

- [22]. Franze K, Grosche J, Skatchkov SN, Schinkinger S, Foja C, Schild D, et al. Muller cells are living optical fibers in the vertebrate retina. *Proc Natl Acad Sci U S A*. 2007; 104:8287–92. [PubMed: 17485670]
- [23]. Chintala SK, Zhang X, Austin JS, Fini ME. Deficiency in matrix metalloproteinase gelatinase B (MMP-9) protects against retinal ganglion cell death after optic nerve ligation. *J Biol Chem*. 2002; 277:47461–8. [PubMed: 12354772]
- [24]. Santos AR, Corredor RG, Obeso BA, Trakhtenberg EF, Wang Y, Ponmattam J, et al. beta1 integrin focal adhesion kinase (FAK) signaling modulates retinal ganglion cell (RGC) survival. *PLoS One*. 2012; 7:e48332. [PubMed: 23118988]
- [25]. Sernagor E, Eglén SJ, Wong RO. Development of retinal ganglion cell structure and function. *Prog Retin Eye Res*. 2001; 20:139–74. [PubMed: 11173250]
- [26]. Wang J, Ye R, Wei Y, Wang H, Xu X, Zhang F, et al. The effects of electrospun TSF nanofiber diameter and alignment on neuronal differentiation of human embryonic stem cells. *J Biomed Mater Res A*. 100:632–45. [PubMed: 22213384]
- [27]. Mahairaki V, Lim SH, Christopherson GT, Xu L, Nasonkin I, Yu C, et al. Nanofiber matrices promote the neuronal differentiation of human embryonic stem cell-derived neural precursors in vitro. *Tissue Eng Part A*. 17:855–63. [PubMed: 20973749]
- [28]. Kador KE, Montero RB, Venugopalan P, Hertz J, Zindell AN, Valenzuela DA, et al. Tissue engineering the retinal ganglion cell nerve fiber layer. *Biomaterials*. 2013; 34:4242–50. [PubMed: 23489919]
- [29]. Kador K, Goldberg J. Scaffolds and Stem Cells: Delivery of Cell Transplants for Retinal Degenerations. *Expert Reviews of Ophthalmology*. 2012 Accepted 8/1/12.

Fig. 1. General experimental procedures. (A) Simplified chemical reaction scheme for the chemically cross-linked hydrogels used in the *in vitro* studies. (B) Schematic showing the hydrogel preparation procedures and experimental plan for cell seeding and analysis.

Fig. 2. Hydrogel morphology. (A) SEM image of a cross section of swollen E4b hydrogel showing the porous nature of the gel. (B) Higher magnification SEM image of swollen E4b hydrogel showing a network of pores approximately 200 μm and smaller.

Fig. 3.

RGCs and ACs prefer specific hydrogels. RGCs and ACs were seeded onto hydrogels and stained using calcein-AM (green) and propidium iodide (red) to mark living and dead cells, respectively. (A) RGCs on a hydrogel (D1c) that poorly supports survival. Note the high proportion of dead (red) cells. (B) RGCs on a hydrogel (E4c) that increases the proportion of surviving (green) cells. (C-D) RGC (C) and amacrine cell (D) neurite outgrowth is elaborate over the surface of the E3b hydrogel. (E) Column graph of the percent survival of RGCs and ACs on laminin-coated hydrogels. Data represented as mean $\pm$ SD. (Scale bar, 50 μ m)

Fig. 4.

RGCs and ACs cells migrate into hydrogels and readily extend elaborate neurites in three dimensions. RGCs and ACs were seeded onto hydrogels and stained using calcein-AM (green) to label cell somas and neurites. Live confocal z-stack images were acquired. RGCs (A) and ACs (B) retained their stereotyped morphology. (D) Percent of cells capable of extending at least one neurite over 200 μm. (Scale bar, 50 μm for A-B, 200 μm for C).

Fig. 5. RGC neurites grow in random directions on hydrogel scaffolds. (A) RGC neurites were imaged and assigned net orientations (ellipses; see Methods). (B) The frequency of neurites extending in each direction, partitioned into three bins of 30°. There was no significant difference between bins, indicating a lack of directionality to RGC neurite growth. (C) The Shannon entropy of the θ distribution from the histograms (see Methods) was high, indicating random orientation, but did not differ between hydrogel scaffolds. (Scale bars, 200 μm)

Table 1

PEG/PLL hydrogels analyzed for RGC and AC survival.

NH ₃ :OH	6:1		4:1		3:1		1:1	
	PLL (MW in kDa)		PLL (MW in kDa)		PLL (MW in kDa)		PLL (MW in kDa)	
4-arm PEG (Mn g/mol)	(b) 70-150	(c) 150-300	(b) 70-150	(c) 150-300	(b) 70-150	(c) 150-300	(b) 70-150	(c) 150-300
(D) 2000	D6b	D6c	D4b	D4c	D3b	D3c	D1b	D1c
(E) 10,000	E6b	E6c	E4b	E4c	E3b	E3c	E1b	E1c

The cells shaded in grey indicate hydrogels that do not polymerize.

Table 2

PEG/PLL hydrogels analyzed for RGC and AC survival (++ survival >70%; + survival 50-70%; – survival <50%).

Hydrogel	NH ₃ :OH	4-arm PEG (Mn g/mol)	PLL (MW in kDa)	Avg. Elastic Modulus (Pa) [range]	RGC survival	AC survival
D1b	1:01	2000	70-150	1406 [1191-1666]	+	+
D1c	1:01	2000	150-300	2961 [2184-3010]	–	–
E3b	3:01	10000	70-150	3843 [3843-3988]	++	++
E3c	3:01	10000	150-300	4309 [4151-4479]	++	+
E6b	6:01	10000	70-150	4330 [3386-5346]	–	–
E6c	6:01	10000	150-300	4988 [4249-5548]	–	–
E4b	4:01	10000	70-150	5057 [4643-5315]	++	++
E4c	4:01	10000	150-300	5730 [5515-5794]	++	+
D6b	6:01	2000	70-150	6381 [2770-10417]	–	–
D4b	4:01	2000	70-150	6888 [6461-7461]	–	–
D6c	6:01	2000	150-300	7774 [4383-9946]	–	–
D3c	3:01	2000	150-300	16197 [12790-25734]	+	+
D3b	3:01	2000	70-150	20132 [14239-27232]		
D4c	4:01	2000	150-300	23146 [20934-26124]	–	–