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Semi-synthetic hydrogel composition and stiffness regulate neuronal morphogenesis

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ABSTRACT

This study describes the use of a set of protein-based biomaterials that allow us to explore the mechanism of cell-mediated 3-D invasion associated with peripheral nerve regeneration. Hydrogels made from poly (ethylene glycol) (PEG) conjugated extracellular matrix proteins, including fibrinogen, gelatin and albumin were compared in their ability to support the neurite extension and glial cell migration from dorsal root ganglion (DRG) as compared to PEG only hydrogel controls. The synthetic polymer in the system provides a cross-linked network with controlled mechanical properties and degradation, whereas the protein components provide the unique extracellular matrix (ECM) for controlling neuronal cell morphogenesis. A range of hydrogel compositions were found to support DRG cell outgrowth, based on the mechanical properties, density and proteolytic degradation of the matrix. The 3-D invasion and morphogenesis of newly grown neurites and glial cells in the different materials were characterized and correlated to the properties of the scaffolds. The DRG cell outgrowth was highly correlated with the density of different hydrogel compositions.

1. Introduction

Three-dimensional cell culture scaffolds are not only prevalent in tissue engineering, they are also aiding in new techniques that allow investigating and controlling the behavior of cells *in vitro*. In neuronal tissue engineering, the regulation over cell behavior using customized biomaterials can be utilized to stimulate tissue formation and enhance peripheral nerve regeneration (Wang and Cai, 2010; Stoll et al., 2002; Stang et al., 2005; Spivey et al., 2012; Siemionow et al., 2013; Schmidt and Leach, 2003a; Scheib and Höke, 2013; Nectow et al., 2012; Mukhatyar et al., 2009; Madison et al., 1987; Lee et al., 2008; Hudson et al., 1999; Haycock, 2011; Gunn et al., 2005; Evans, 2001; Drury and Mooney, 2003; Deumens et al., 2010; de Ruiter et al., 2009; Daly et al., 2012; Cullen et al., 2011a; Bell and Haycock, 2012). For example, biomaterials can be designed as neuronal scaffolds to mimic signals that direct natural nerve tissue development *in vivo*, including neurotrophic factors, extracellular matrix interactions, and cell-cell communications (Drury and Mooney, 2003; Daly et al., 2012; Sherman and Brophy, 2005; Lee and Wolfe, 2000). Beyond their utility as nerve guidance conduits (NGCs), these biomaterial systems provide a good platform to study and control the mechanism of nerve tissue formation *in vitro*. In this research, we employ an *in vitro* model of nerve repair to investigate a tissue engineering strategy premised on semi-synthetic biomaterials that enable precisely controlled environmental stimuli to regenerating neurons and glial cells.

A number of different natural and synthetic biomaterials – together with well-defined fabrication techniques – have been used to prepare neural scaffolds as NGCs. Some of the scaffold materials include collagen, fibrin, agarose, agarose derivatized with Laminin-1 or LN-1 peptides, Matrigel, PCL, PGA, PEG and polyHEMA (Deumens et al., 2010; Bellamkonda, 2006; Kriebel et al., 2014; Kehoe et al., 2012; Pittier et al., 2005). Typically, these materials are fabricated with different internal architecture or surface patterns in order to create a microenvironment for guiding axonal bundle regeneration (Nectow et al., 2012; de Ruiter et al., 2009; Bellamkonda, 2006; Cullen et al., 2011b; Gu et al., 2011; Xie et al., 2014; Lu et al., 2014; Zeng et al., 2011).

The desired performance of a scaffold may be accomplished by harnessing natural nerve repair pathways. The biological events that mediate nerve tissue repair are regulated by the provision of degradation factors that remove the damaged debris and biological factors that facilitate the regrowth of the new tissue. Support of

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extracellular matrix (ECM) proteins, such as collagen – a main component of nerve connective tissue structure – and growth factors that enhance the regeneration of the new nerve tissue have been elucidated to play a role in natural nerve repair (Stang et al., 2005; Shoulders and Raines, 2009; Mouw et al., 2014; Möllers et al., 2009; Chen et al., 2014; Yoshii and Oka, 2001; Herbert et al., 1998; Alovskaya et al., 2007). Indeed, when the nerve structure remains intact after an injury, axon regeneration is possible through the existing perineurium sheath. Nerve regeneration progresses in hollow nerve conduits that replace damaged nerve structure, is initiated by influx of plasma exudate from both the proximal and distal nerve stumps. The exudate is enriched with neurotrophic factors and extracellular matrix (ECM) precursor molecules (e.g. fibrinogen and factor XIII). This is followed by the formation of an acellular fibrin cable between the proximal and distal stumps, formed from the influxed ECM precursor molecules and restricted by the gap size between the two nerve stumps (Schmidt and Leach, 2003b; Kalbermatten et al., 2008; Pettersson et al., 2010; Belkas et al., 2004). This fibrin cable usually forms within one week of repair and forms an ECM bridge for the next stages of regeneration.

Next, Schwann cells (SCs) from the proximal and distal nerve stumps, as well as some endothelial cells and fibroblasts that produce collagen, migrate along this fibrin cable. These SCs subsequently proliferate and align, forming an aligned SC cable, i.e. the glial bands of Büngner. This biological tissue cable provides a trophic and topographical tissue cable for the axonal phase of repair. During this axonal phase, new regenerative axonal sprouts, guided by their individual growth cones, use this biological cable tissue as a guidance mechanism to ultimately reach their distal target (Daly et al., 2012; Belkas et al., 2004). However, the sustained presence of fibrin or collagen in the injury site can also interfere with the delicate timing of the repair process and disrupt the construction of functional nerve, resulting instead in the formation of a scar tissue. Therefore, the ability to control the structure, composition and degradation rate of the NGC's provisional biomaterial scaffold is crucial for enabling successful nerve regeneration using natural repair pathways. Although fibrin and collagen based biomaterials have been used extensively to create NGCs, these materials lack the ability to precisely control the degradation properties of the scaffold during the regeneration process. In this regard, it is very difficult to apply the structural and inductive features of reconstituted natural protein-based biomaterials for regulating tissue regeneration.

Semi-synthetic scaffolds – particularly those based on protein-polymer adducts – can provide much better temporary structural support for cells to grow and regenerate, as well as spatial and temporal control over cell signaling molecules that regulate the regeneration processes (Nectow et al., 2012; Halstenberg et al., 2002; Almany and Seliktar, 2005). Additionally, the ability to control the composition and degradation rate of the nerve guiding provisional biomaterial scaffold is a critical requisite for enabling successful nerve regeneration using natural repair pathways. We have focused our research efforts on creating semi-synthetic provisional nerve scaffolds comprised of natural fibrinogen, gelatin, or albumin and synthetic poly(ethylene glycol) (PEG) constituents. Fibrinogen is a soluble precursor molecule of fibrin; hence it presents a highly relevant biological microenvironment for axonal growth (Pittier et al., 2005; Dubey et al., 2001; Schense et al., 2000; Ryu et al., 2009; Kamath and Lip, 2003; Barker et al., 2001). Gelatin is a partial derivative of collagen, formed by breaking the natural triplehelix structure of collagen into single-strand molecules by hydrolysis. Gelatin is less immunogenic compared to its precursor, and presumably retains bioactive signals such as the RGD sequences, thus promoting cell adhesion, migration, differentiation, morphogenesis, and proliferation (Tan and Marra, 2010). The albumin protein serves as a biological agent with no specific affinity, yet with proteolytic susceptibility. This combination allows us to control the degradation rate, protein composition, and structural features of the matrix and thus to establish a more precise platform for the *in vitro* and *in vivo* regeneration of peripheral nerves. In this study, we describe the production of three different scaffold materials: PEG-Fibrinogen, PEG-Gelatin, and PEG-Albumin hydrogels. We demonstrate the capability of neonatal chick dorsal root ganglia (DRGs) encapsulated into the materials in supporting neurite and Schwann cell invasion in a 3-D manner. We further demonstrate that altering the matrix properties (protein composition, degree of cross-linking, and degradation rate) results in differentiated 3-D invasion kinetics and morphogenesis of neuronal cells into the encapsulating semi-synthetic scaffolds.

2. Materials and methods

2.1. Fibrinogen and albumin PEGylation

PEG-diacrylate (PEG-DA) was prepared from linear PEG-OH MW = 10 kDa (Fluka,Buchs, Switzerland) as described elsewhere (Almany and Seliktar, 2005). The conjugation of PEG to fibrinogen or albumin (i.e. PEGylation) was performed according to a protocol similar to the one described in detail by Dikovsky et al. (Dikovsky et al., 2006). Briefly, Tris (2-carboxyethyl) phosphine hydrochloride (TCEP-HCl) (Sigma-Aldrich) was added to a 7 mg/ml solution of bovine fibrinogen (MP Biomedicals, Solon, OH, USA) or bovine serum albumin (MP Biomedicals, Ohio, USA) in 150 mM phosphate buffered saline (PBS) with 8 M Urea (molar ratio 1.5:1 or 1:8.7 TCEP to fibrinogen or albumin cysteines, respectively). Linear 10 kDa PEG-DA was reacted for 3 h or overnight with the proteins at a 4:1 or 2:1 molar ratio of PEG to fibrinogen or albumin cysteines, respectively. The PEGylated protein product was precipitated in acetone and redissolved in PBS containing 8 M urea to 10–12 mg/ml final protein concentration. The protein product was dialyzed against PBS at 4 °C for 1 d (Spectrum, 12–14 kDa MW cutoff, California, USA). The PEGylated fibrinogen product was sterilized and characterized according to previously published protocols (Dikovsky et al., 2006). Briefly, the protein concentration was determined using a BCA™ Protein Assay (Pierce Biotechnology, Inc., Rockford, IL). To establish the total PEG-protein concentration, 0.5 ml of the precursor solution was lyophilized overnight and weighed. The amounts of total PEGylated product (dry weight) and protein content (BCA™ result) were used to calculate the PEG concentration in the precursor solution. The final desired protein concentration (8 mg/ml) was achieved by diluting the PEGylated protein precursor with PBS. To increase the cross-link density of the hydrogel, a linear 10 kDa PEG-DA was added to the solution to reach desired final PEG concentration. The formation of a hydrogel was accomplished by photo-polymerization of the PEGylated protein solution (with or without additional PEG-DA) using a 0.1% (w/v) photo-initiator, Irgacure®2959 (Ciba Specialty Chemicals, Basel, Switzerland/Tarrytwon, New York) and long-wave UV light (365 nm, 4–5 mW/cm^2) exposure for 5 min.

2.2. Gelatin PEGylation

Thiolation of gelatin (Gelatin A, Porcine skin, Sigma) was accomplished using succinimidylacetyl-thioacetate (SATA, Pierce, Illinois, USA) based on the protocols described by Chen et al. (Chen et al., 2002). Briefly, gelatin was dissolved in 150 mM PBS with 8 M urea at a concentration of 7 mg/ml. SATA was reacted for 2 h at pH 7.5 and 37 °C with agitation (molar ratio 61:1 SATA to gelatin). The resulting acetylated SH groups on the lysine residues were then deprotected by reacting 0.5 M hydroxylamine hydrochloride