A Micro/Nano Particle Protein Delivery System for Axon Regeneration in Spinal Tissue

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Statement of Purpose: Following a spinal cord injury, axon regeneration in spinal tissue is difficult for various reasons. Two main reasons include the lack of growth factors as well as the presence of physical barriers caused by glial scar formation. Peripheral nerve grafts (PNG) have been used to bypass the injury sight and provide a bridge for axon growth (1). The focus of this work is on the use of micro and nano particles as a delivery system for both a type of growth factor (Brain Derived Neurotrophic Factor – BDNF) and a scar formation inhibitor (Chondroitinase). The desired delivery period of bioactive BDNF is up to 2-4 weeks and Chondroitinase is up to 4 days. The delivery of BDNF is to provide a concentration gradient to entice axon growth out of the PNG. The purpose of Chondroitinase is to prevent scar formation when the PNG is attached to the spinal tissue. For these studies lysozyme was used as a model protein. Particles are composed of poly(lactic-co-glycolic acid) (PLGA) alone or a blend of PLGA and sucrose acetate isobutyrate (SAIB). They are synthesized using water-oil-water (W-O-W) double emulsion solvent evaporation to allow for the incorporation of proteins. Research has focused on the development of this particle protein delivery system and how variations in the fabrication process affect particle size, protein loading, protein release and protein bioactivity. Fluoresced PLGA particles have also been injected into rat spinal tissue and analyzed for biodistribution, macrophage activity and glial scar formation.

Methods: The effect of the W-O-W double emulsion procedure on particle size and loading was measured by varying the following: type of solvent (Dichloromethane (DCM), Acetone or Ethyl Acetate); the mixing speed (homogenization at 10,000 or 25,000 rpm or sonication (son)); and the concentration of PLGA in the organic phase (10mg/ml or 30mg/ml). Particle size was measured by Scanning Electron Microscopy (SEM) and lysozyme loading was determined both directly (dissolving particles in DCM and extracting protein into water) and indirectly (analyzing the amount of protein in the supernatant after centrifuging of particles). The effect of the synthesis procedure on protein stability and activity was determined by subjecting the protein to the synthesis procedure without the presence of PLGA. The following variables were analyzed: type of solvent (DCM, Acetone or Ethyl Acetate); the mixing speed (homogenization at 10,000 or 25,000 rpm or sonication); and the use of methoxy poly(ethylene-glycol) (mPEG) as a stabilizer (50mg/ml MW 5,000 Da). Following solvent evaporation, samples were centrifuged to remove precipitated lysozyme and the supernatant analyzed for concentration and activity. To determine the effect of particle size and an additive (SAIB) on protein release, particles were synthesized using the W-O-W procedure and the following varied: solvents (DCM, Acetone, Ethyl Acetate); mixing speed (homogenization

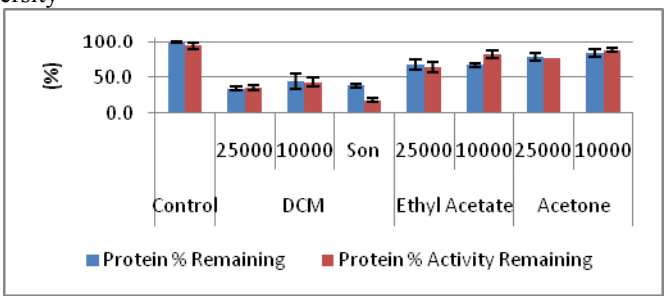

Figure 1. Effect of Particle Synthesis on Lysozyme Precipitation and Activity

25,000 rpm or sonication); PLGA alone or PLGA and SAIB (5:1 wt ratio). At various time points, particles were centrifuged and the supernatant collected for concentration and activity analysis. In all cases, lysozyme concentration was measured by the BCA Assay and activity was measured by the EnzChek ® Assay. Particle biodistribution and tissue response was measured by making variable size fluoresced particles and injecting 1 ul (0.4 mg/ml) of particles into rat spinal tissue at both injured (tissue aspiration) and uninjured sights. Rat spinal cord was extracted 7 days after injection and immunostained for macrophages and scar formation. Analysis was performed under fluorescent microscopy.

Results: The effect of the W-O-W procedure on particle size and loading indicates that particle size decreases with increasing solvent miscibility. The less miscible the solvent, the greater the effect that mixing speed has on size and increased agitation results in decreased particle size. Increased agitation also results in increased loading. Increasing PLGA concentration has the maximum effect on loading efficiency with minimal effect on the protein weight percent of the particle. The effect of the synthesis procedure on lysozyme is indicated in Figure 1. It shows that the type of solvent has the most effect on protein precipitation with DCM being the most damaging. MPEG as a stabilizer has little effect. In general, if lysozyme remains in solution than it maintains its activity. Because of protein precipitation loading efficiency is dependent on whether loading is measured indirectly or directly. Release data indicates variable delivery rates with delivery of active protein up to several weeks. In-vivo work shows that the particles generally stay at the sight of injection and cause no noticeable scar formation. A minimal macrophage response is detected, but it is unclear if the response is caused from the injection or from the particles.

Conclusions: Results indicate that the nano and micro particle system researched is an acceptable delivery system for both BDNF and Chondroitinase. Future work involves the loading of BDNF and Chondroitinase into particles and performing release studies to both measure release rates and verify activity. Future work also involves injecting Chondroitinase and BDNF loaded particles into spinal tissue and measuring axon growth.

References: (1) Tom V. Houle J. Exp Neurology. 2008;211:315-319