Neuron-specific Polymeric Micelle as a siRNA Delivery Carrier for CNS Regeneration

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Statement of Purpose:

Adult CNS axons retain the intrinsic capability for growth, but fail to do so effectively following injury due to the presence of native and injury-induced growth inhibitors in the extracellular environment. Three molecules present in CNS myelin (Nogo A, myelin associated glycoprotein, and oligodendrocyte myelin glycoprotein) have been identified as inhibitors of axonal growth. All three molecules have been shown to bind to a common receptor called the Nogo-66 receptor (NgR1)[1]. The long-term objective of this project is to develop neuron-specific polymeric micelles as a carrier for simultaneous delivery of two therapeutic agents (antibody and siRNA) directed at reducing myelin-based inhibition of axonal regeneration. This dual strategy is based on interference with the function of existing NgR1 receptors at the protein level and inhibition of further production of new NgR receptors at the genetic level[2]. We have previously described the synthesis and evaluation of a novel amphiphilic co-polymer, poly (lactide-co-glycolide)-graft-polyethylenimine (PLGA-g-PEI:PGP), as a nucleic acid carrier using pGFP [3]. Here, we demonstrate the feasibility of PGP as a siRNA delivery carrier using GFP siRNA and siGLO® transfection indicator.

Materials and Methods:

1. Transfection efficiency of PGP/pGFP complex in serum -free and serum conditions in primary neurons:
Primary E8 chick forebrain neurons (CFNs) were prepared as previously described[4] and plated in 12-well plates (0.9~1.1 x 10^6 cells/well) pre-coated with 0.1 % PLL. PGP/pGFP (Promega) complexes (2 μg of pDNA) were prepared at varying N/P ratios and the cells were transfected in serum-free media and in 10% serum media. After 48hrs post-transfection, cells were fixed and quantitative cell counts were performed to measure transfection of CFNs.

2. Cytoxicity of PGP/pGFP complex in CFN neuron cells:
To evaluate cytotoxicity, parallel experiments were performed as described above. After 48 hours transfection, the cytotoxicity was analyzed by MTT assay.

3. Evaluation of PGP as a siRNA delivery carrier: To verify PGP as a siRNA delivery carrier, PGP/siGLO Red (Thermo Fisher Scientific) complexes were prepared at N/P ratio of 30/1 and transfected in B35 cells and E8 CFNs. At 4hrs post-transfection, cells were fixed, counter stained by DAPI, and digitally imaged.

4. Gene silencing efficiency of PGP/GFPsiRNA in vitro
PGP/GFPsiRNA (Ambion) complexes at varying N/P ratios (0.25 μg of siRNA) were co-transfected to B35 cells with PEI/pGFP. Briefly, cells were transfected with PEI/pGFP complex at N/P ratio of 5/1 (2 μg of pGFP) in serum-free conditions as described above. At 24 hrs post-transfection, PGP/GFPsiRNA complexes were transfected to PEI/pGFP transfected B35 cells. The level of GFP expression was measured and all values presented were normalized by protein content determined by BCA assay.

Results and Discussion

1. Transfection efficiency of PGP/pGFP complex: The percent transfection of PGP /pGFP complexes was assessed in CFN cells at varing N/P ratios (Fig 1). PGP/pGFP complexes at N/P ratios of 10/1 showed similar transfection efficiency compared to PEI in serum-free condition, while PGP/pGFP complexes at N/P ratios of 35/1 showed 1.42 times higher transfection efficiency compared to PEI in 10% serum free condition.

2. Cytotoxicity of PGP/pGFP complex in vitro: The percent cell viabilities of CFNs transfected with PGP/pGFP at N/P ratios of 10/1 and 15/1 were about 59 and 54.8 %, respectively, and showed higher cell viability than that of PEI/pGFP at N/P of 5/1 (45.6 %) in serum–free condition, while cell viabilities of CFNs transfected with PGP/pGFP at all N/P ratios were similar with PEI control in 10% serum condition (Fig 2).

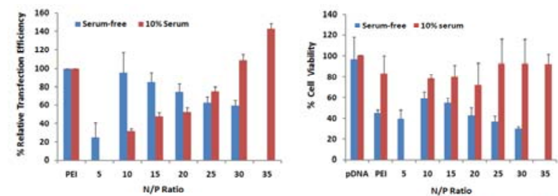

Figure 1. GFP expression in E8 CFN cells after transfection in seum-free and 10% serum. PEI/pGFP complex (N/P: 5/1) was used as control. Data represent the mean ±STD (n=12).

Figure 2. Cell viability in E8 CFN cells after transfection in seum-free and 10% serum. Naked DNA and PEI/pGFP (N/P: 5/1) was used for comparison.

3. Evaluation of PGP as a siRNA delivery carrier:
To evaluate PGP as a siRNA delivery carrier, siGLO® Red transfection indicator was used. PGP/siGLO at N/P of 30/1 showed greater than 90% transfection efficiency (Fig 3).

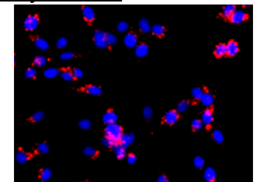

Figure 3. PGP/siGLo Red (N/P :30/1) transfected B35 cells. Red shows siGlo Transfected cells and blue shows DAPI stained nucleus. Original magnification, 400X

4. Gene silencing efficiency of PGP/GFPsiRNA in vitro:
The level of GFP expression obtained from PEI/pGFP (control) transfection was defined as 100%. Co-transfection with PGP/GFP siRNA complexes at N/P ratio 10/1, 25/1 and 30/1 showed 58.5, 85.4, and 89.8 % silencing efficiency and showed higher silencing than that with ExtremeGene /GFP siRNA complex(5/1 ratio: 34.5%).

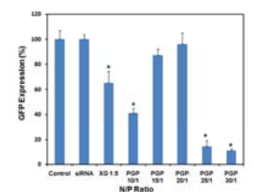

Figure 4. Percent silencing efficiency with PGP/GFP siRNA complexes in B35 cells after co-transfection with PEI/pGFP complex at N/P ratio of 5/1 (n=6). *P<0.05

Conclusions: We demonstrated that PGP is a promising siRNA delivery carrier with high gene silencing efficiency after co-transfection of pGFP using PEI. Currently, we are preparing PLGA-g-PEI-Ab (Ab:NgR antibody) and evaluating the feasibility of PLGA-g-PEI-Ab as a neuron-specific nucleic acid (NgR siRNA) carrier for CNS regeneration in rat DRG/astrocyte co-culture system.

References : 1. Fournier et al. *Nature*, **409**, 341-6 (2001) 2. Ahmed et al. *Mol Cell Neurosci*, **28**, 509-23 (2005) 3. Lee et al. *Trans SFB* p.917 (2010). 4. Cribb et al. *Prot Express Purif*, **57**, 172-9 (2008).

Acknowledgments: SC Center of Biomaterials for Tissue Regeneration and NIH grant #P20RR021949.

Abstract #681
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