Polyethylene glycol-based Protein Nanocapsules for Functional Delivery of a Differentiation Transcription Factor

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Statement of Purpose: Transcription factors (TFs) are modular proteins that include one or more DNA binding domains capable of attaching to specific sequences and regulating transcription of specific genes. Controlling intracellular levels of specific TFs is a powerful method to redirect cellular fate and produce healthy functioning cells of desired lineages. Developing tools to directly deliver TFs to specific cells can therefore have widespread therapeutic value in regenerative medicine. We have developed polymeric protein nanocapsules (NCs) to facilitate intracellular delivery of diverse proteins. To achieve intracellular release of protein, crosslinkers are designed to be degradable only when inside the cells. We used a peptidyl crosslinker containing a highly favored substrate (RVRR) of furin, a ubiquitous endoprotease in mammalian cells. Another degradation strategy is by using a redox-responsive, disulfide containing crosslinker *N,N'*-bis(acryloyl)cystamine. Here we demonstrate the synthesis, optimization and application of poly(ethylene) glycol (PEG) based protein NCs in the nuclear delivery of the TF MyoD for differentiation of myoblasts into myotubes.

Methods: PEG NCs were synthesized using a short chain length PEG (M_n~480), a positively charged co-monomer N-(3-aminopropyl) methacrylamide and furin-degradable or redox-responsive crosslinkers. NCs were fabricated using adsorption of monomers and crosslinkers to protein, and subsequent *in situ* polymerization. PEG NCs were optimized using nuclear imaging and fractionation to determine protein content. MyoD protein/NCs were delivered to C2C12 myoblast cells using the protocol shown in Figure 1A. Cells were immunostained using a myosin heavy chain antibody and RNA was extracted for qPCR analysis of myogenic genes.

Results: Physical properties and release kinetics of PEG NCs were optimized through tuning of monomer and crosslinker ratios to achieve enhanced delivery of cargo destined for the nuclei. The NCs did not display cytotoxicity in primary cell lines up to concentrations of 5 μM. A recombinant myogenic transcription factor, MyoD, was delivered to the nuclei of myoblast cells using degradable NCs to induce myogenic differentiation. MyoD was confirmed to be delivered to the nuclei of myoblasts using confocal microscopy and was demonstrated to be active in transcription through a luciferase-based reporter assay. More importantly, delivered MyoD was able to drive myoblast differentiation as evidenced by the hallmark elongated and multinuclear morphology of myotubes. (Figure 1) The activation of downstream cascade was also confirmed through immunostaining of late myogenic markers myogenin and My-HC. The efficiency of differentiation achieved via NC delivery is significantly higher than that

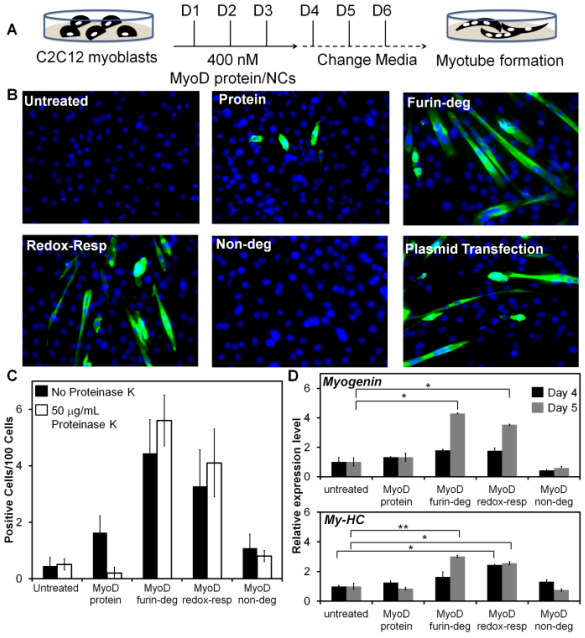

Figure 1. C2C12 myoblasts can be differentiated into myotubes using degradable MyoD NCs. (A) Schematic of treatment of C2C12 myoblasts with MyoD protein/NCs to induce differentiation to multinucleated, elongated myotubes. (B) Fluorescent images of C2C12 cells after MyoD protein/NC treatment. Cells were immunostained with a myosin heavy chain (My-HC) antibody conjugated with an Alexa-Fluor 488 antibody (green). Nuclei were counterstained with DAPI (blue). *(C)* Quantification of positively-stained cells for each treatment group with and without 50 μg/mL proteinase K for 1 h at 37°C. Data is the average and standard deviation of 10 images. *(D)* Real-time PCR analysis of the relative expression of myogenin and My-HC in treated C2C12 cells at day 4 and day 5 of treatment. (n=2). Unpaired student *t*-test; $^{*}P<0.05$; $^{**}P<0.01$.

of native MyoD, and is comparable to that of plasmid transfection. The encapsulated MyoD can also withstand prolonged protease treatment and remain functional.

Conclusions: In this study, we demonstrated the design, synthesis and optimization of PEG-based degradable protein NCs. The degradable NCs can subsequently deliver MyoD to the cytosol of myoblast cells, release functional MyoD to enter the nuclei and initiate myogenic differentiation. Importantly, PEG NCs are validated as a platform which retains encapsulated protein structure and activity as evidenced by the ability of MyoD to perform complex downstream processes and regulate myogenesis. The ease of preparation, biocompatibility and effective cargo delivery make the polymeric NC a useful tool to deliver a variety of recombinant TFs for therapeutic uses.

References: 1) Gu et al. Nano Lett. 2009;9:4533-8, 2) Biswas et al., ACS Nano. 2011;5: 1385-94, 3) Zhao et al. Biomaterials. 2011;32:5223-30, 4) Biswas et al., Biomaterials. 2012; 33:5459–67.