Intracellular Trafficking of Alginate-graft-Poly (Ethylene Glycol) Microspheres for Promoting Mesenchymal Stem Cell Osteogenic Differentiation

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Statement of Purpose: Osteoporosis is a skeletal fragility associated with reduced quantity and quality of bone, and is a major problem in the aging population. The pathogenesis of the disease arises from a dysfunction in the differentiation pathway of mesenchymal stem cells (MSCs). MSCs from osteoporotic patients are more likely to differentiate into adipocytes than osteoblasts compared to cells isolated from patients with normal bone mass. Intracellular delivery of vascular endothelial growth factor A (VEGFA) enhances osteogenesis of MSCs—a possible pathway to accelerate bone mineralization.[1] In a previous study we demonstrated the ability to use alginate-based microspheres to intracellularly deliver VEGFA and promote MSC osteogenic differentiation.[2] The goal of this study was to evaluate the efficiency of internalization of VEGFA-encapsulated microspheres by MSCs to provide a better understanding of drug treatment efficacy for further clinical applications. We will evaluate both alginate-*graft*-poly(ethylene glycol) (Alg-*g*-PEG) microspheres and alginate-*graft*-poly(ethylene glycol)-S-S-RGD (Alg-*g*-RGD) microspheres *in vitro* using confocal laser scanning (CLS) microscopy and flow cytometry.

Methods: The preparation of Alg-*g*-PEG and Alg-*g*-RGD microspheres were based on the literature by Miao *et al.* (2014).[2] Briefly, 2,2'-dithiodipyridine was conjugated onto amine-poly(ethylene glycol)-thiol (NH_2-PEG-SH, MW=1000g/mol).[3] Alg-g-PEG copolymers were synthesized via carbodiimide chemistry. Alg-g-PEG solutions was mixed with VEGFA then formed into microspheres using a water/oil emulsion and the addition of calcium chloride. Cysteine-RGD was conjugated onto the pyridine end of Alg-g-PEG and covalently conjugated onto the surface of microspheres via a disulfide bond. An additional step of carbodiimide chemistry was performed between the Alg-*g*-PEG and Alg-*g*-RGD microspheres and Alexa Fluor® 647 Cadaverine (Life Technology Inc.) catalyzed by N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC). CLS microscopy was used to visualize intracellular transport of VEGFA-encapsulated microspheres into primary human MSCs after 48 h of culture and 10 minutes Hoechst 33342 nuclei stain. Flow cytometry was applied to determine the population of encapsulated microspheres within MSCs after 48 h of culture. Cells were rinsed with fresh medium prior to analysis to remove non-internalized microspheres.

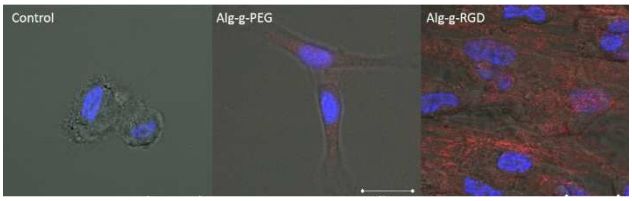

Figure 1 CLS images of primary human MSCs with Alexa Fluor® 647 labeling Alg-*g*-PEG and Alg-*g*-RGD microspheres (red); nuclei were stained with Hoechst 33342 (blue).

Results and Discussion: The CLS micrographs are shown in Figure 1. The Alexa Fluor® 647 labeled microspheres (red color) are seen within the cytoplasm of MSCs (blue = nuclei). Alg-*g*-RGD displayed a stronger signal compared to Alg-*g*-PEG. Flow cytometry was first applied to determine the labeling efficacy of Alexa Fluor® 647 and 90% of the microspheres were successfully labeled with Alexa Fluor® 647; no auto fluorescence was seen for non-labeled microspheres (Figure 2A). Figures 2B and 2C are comparisons of cells treated with unlabeled microspheres (red) and cells treated with Alexa Fluor® 647 labeled microspheres (blue). The shift between red and blue populations indicates that Alexa Fluor® 647 labeled microspheres were internalized by MSCs. Differences between the red and blue population peaks suggest that Alg-*g*-RGD microspheres were more readily internalized within MSCs compared to Alg-*g*-PEG microspheres.

Conclusion: By utilizing fluorescent labeling of Alg-*g*-PEG and Alg-*g*-RGD microspheres, we were able to determine cellular internalization efficiencies. The surface-conjugated ligand peptide RGD increased microsphere internalization, providing a better strategy for intracellular VEGFA delivery for osteoporosis treatment. Additional assays are ongoing to determine the location of entrapped microspheres. Fluorescently-labeled microspheres may also be applied as imaging probes for various applications.

References: 1. Liu *et al. Journal of Clinical Investigation* (2012); 2. Miao *et al. Journal of Controlled Release* (2014)

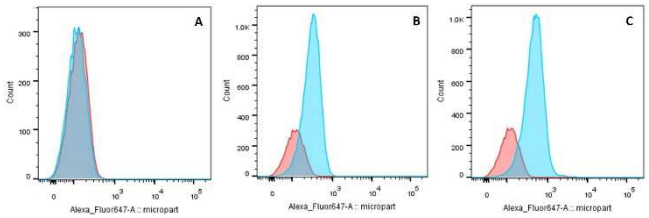

Figure 2 Flow cytometry analysis of Alg-*g*-PEG and Alg-*g*-RGD microsphere internalization within MSCs. (A) Red is pure MSCs without any microsphere treatment; blue is MSCs treated with non-labeled microspheres. (B) Red is MSCs treated with non-labeled microspheres; blue is MSCs treated with Alexa Fluor 647 labeled Alg-*g*-PEG microspheres. (C) Red is MSCs treated with non-labeled microspheres; blue is MSCs treated with Alexa Fluor 647 labeled Alg-*g*-RGD microspheres.