High-efficiency, Low-toxicity Nanopieces Designed for siRNA Delivery in Extracellular Matrix-rich Musculoskeletal Tissues

Yupeng Chen¹, Kaitlyn Tracy¹, Hongchuan Yu¹, Hicham Fenniri²,³, Qian Chen¹.
¹Brown University/Rhode Island Hospital, Providence, RI, USA, ²Northeastern University, Boston, MA, USA, ³Qatar Biomedical Research Institute, Doha, Qatar.

Disclosures: Y. Chen: None. K. Tracy: None. H. Yu: None. H. Fenniri: None. Q. Chen: None.

Introduction: Since the discovery of RNA interference (RNAi), the use of small interfering RNA (siRNA) to silence disease-inducing genes has shown great therapeutic potential due to its high specificity and efficiency in silencing target gene expression. However, a major challenge in this approach is that siRNA must infiltrate tissue and enter target cells to be effective. This is especially challenging for extracellular matrix-rich musculoskeletal systems. The second challenge is cytotoxicity of the current transfection vehicles. Lipid-based or lipid-composited commercial transfection reagents, such as Lipofectamine 2000™, X-tremeGENE™ and Nanojuice™, show high levels of cytotoxicity upon transfection, which can induce a deleterious pro-inflammatory response and even apoptosis due to their lipid nature. Moreover, they are large in size, forming spheres of several hundred nanometers in diameter (after loading with RNAs), very difficult to penetrate matrix-rich musculoskeletal tissues. Therefore, we have developed a novel delivery vehicle, Nanopieces, consisting of co-assembled rosette nanotubes (RNTs) and siRNA via completely non-covalent bindings (Figure 1). Thus, Nanopieces showed superior biodegradability. Once Nanopieces delivered their siRNA cargo, their degradation products are highly biocompatible due to the biomimetic G^C base motif of the RNT. The ability of Nanopiece to deliver cargo effectively and degrade safely allows minimal levels of cytotoxicity, a prerequisite for in vivo therapeutic applications. Furthermore, Nanopieces have a nano-rod-like shape, 20-30 nm in diameter. This is more than 2000 times smaller in volume than Lipofectamine spherical particles, allowing the Nanopiece to transfect cells that are shielded by dense extracellular matrix.

In this study, we tested two generations of Nanopieces. One is K1-RNT Nanopiece followed by the second generation TBL (twin base linker)-RNT Nanopiece that has two G^C basepair motifs per functional unit as opposed to a single G^C motif seen in the K1-RNT (Figure 1). The TBL-RNT has a stronger positive surface charge and larger diameter due to the second G^C motif allowing more RNAs to load than K1-RNT. In this study, we showed that both Nanopieces transfect matrix-rich musculoskeletal cells in vitro with higher transfection efficiency and lower cytotoxicity than commercial delivery vehicles.

Methods: Various types of cells (including musculoskeletal cell line, musculoskeletal primary cells and non-musculoskeletal, but matrix-rich cells) were used to compare both cytotoxicity and transfection efficiency between Nanopieces (K1 and TBL) and commercial transfection reagents including Lipofectamine 2000™, X-tremeGENE™ and Nanojuice™. Negative control of untreated cells was used throughout. Cells were transfected with respective transfection reagent with fluorescence labeled siRNA, and then stained with Propidium Iodide (PI) to identify dead or dying cells. Cells were processed via Flow Cytometry to quantify successfully transfected cells (those that are positive for fluorescence) and alive, healthy cells (those negative for PI staining). Flow Cytometry was simultaneously used to sort cells based on size and granulation, showing a population of healthy cells with normal phenotype and a distinct population of cell debris. A transfection score was calculated based on Flow Cytometry data to combine transfection efficiency and cytotoxicity into one number.

Transfection Score = (# cells FITC positive / total cells) × (# cells PI negative / total cells) × 100%

Results: Musculoskeletal primary cells - Mouse Chondrocytes: FACS analysis indicated that, while transfection with Nanojuice...
produced high cell death rate, X-tremeGENE and Lipofectamine produced low transfection rate. In contrast, transfection with Nanopiece (K1, and TBL) produced lower cell death rate and higher transfection rate (Fig. 2A). As a result, Nanopieces showed the highest transfection score (71.8 for K1 and 72.2 for TBL) in comparison to Nanojuice (59.8), X-tremeGENE (49.4) and Lipofectamine (40.3) (Fig. 2B).

Musculoskeletal cell line - ATDC5 mouse chondroprogenitor: FACS analysis demonstrated the presence of cell debris after transfection with commercial transfection reagents, suggesting induced cell death. In contrast, transfected cells by Nanopieces showed normal phenotype in terms of cell size and granulation, and had the highest transfection score at 24, 48, and 72 h.

Matrix-rich, Non-musculoskeletal cells - Astrocytes: When transfected with Lipofectamine, majority of the cells positive for
fluorescence were characterized as cell debris. Nanopieces, especially TBL Nanopieces, showed a significantly greater transfection score than Lipofectamine.

**Discussion:** Cells transfected with Nanopieces sustained healthy cell phenotype whereas transfection with commercial reagents yielded high percentage of cell debris, suggesting high levels of cytotoxicity associated with these reagents. In either cell line or primary chondrocytes, Nanopieces showed the highest transfection score at all time points compared to commercial transfection vehicles. Superior transfection score was a function of both high transfection efficiency and low cytotoxicity. Beyond the musculoskeletal system, Nanopieces showed the highest transfection score in other extracellular matrix-rich cells, namely astrocytes, showing promise for the wide range of target cells for which Nanopiece delivery vehicles may be used. The second generation of Nanopieces, namely TBL Nanopieces, has higher transfection efficiency with only 25% of the dose of K1, thus yielding an even more effective transfection vehicle.

**Significance:** Nanopieces, as a novel nano-material, show great potential as a high efficiency and low toxicity delivery vehicle for therapeutic RNAi treatment. Non-covalent binding ensures their excellent biodegradability and low cytotoxicity; while nano-rod-like shape enabled their superior ability to penetrate extracellular matrix. These properties result in high transfection efficiency, even in extracellular matrix-rich tissues.

**Acknowledgments:** The authors would like to thank Center of Biomedical Research Excellence in Skeletal Health and Repair from NIH for funding.

**References:**