Hybrid Charge-reversed Cationic Exosomes for Advanced Non-Viral Gene Therapy in Osteoarthritis

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INTRODUCTION: A major challenge in translating gene therapy for osteoarthritis (OA) is the lack of safe and effective non-viral delivery systems[1]. Interleukin-1 receptor antagonist (IL-1Ra), which blocks IL-1 receptor signaling and prevents inflammatory activation in chondrocytes, has shown therapeutic potential[2]. While adeno-associated viral vectors have been used to deliver IL-1Ra, their clinical use is limited by safety and immunogenicity concerns[3,4]. Exosomes (Exo), natural cell-derived vesicles, offer a promising non-viral alternative due to their biocompatibility and inherent bioactivity[5]. However, their negatively charged lipid bilayer and relatively large size (30–200 nm) restrict cartilage penetration. Our lab has developed charge-reversed cationic Exos that can penetrate full-thickness early-stage cartilage and deliver genetic cargo to deep-layer chondrocytes[6]. Despite this advantage, Exos have limited capacity for large therapeutic genes such as mRNA and plasmid DNA. In contrast, synthetic liposomes (Lipos) have high gene loading efficiency but suffer from toxicity and poor biocompatibility[7]. To address these limitations, we engineered **Cationic Hybrid Exos (Cationic HyExo)** by fusing Exos with cationic Lipos, combining the high loading capacity of Lipos with the biocompatibility of Exos. Using sonication and extrusion, we generated charge-reversed Hybrid Exos capable of delivering IL-1Ra mRNA to chondrocytes for OA therapy (**Fig. 1A**).

METHODS: Exos were isolated by differential ultracentrifugation and size exclusion chromatography from bovine milk. DOPC/cholesterol Lipos were synthesized by thin-film hydration and fused with Exos by extrusion to generate Hybrid Exos. Cationic Lipos composed of DOTAP/DOPC/cholesterol were similarly prepared and co-extruded with Exos to form Cationic HyExo. Particle size and ζ-potential were measured, and morphology was examined by TEM. Hybridization was validated by fusing green fluorescent–labeled Exos with red fluorescent–labeled Lipos and analyzing dual fluorescence using confocal microscopy. For gene loading, eGFP mRNA and in vitro–transcribed (IVT) IL-1Ra mRNA were loaded into Hybrid Exos and Cationic HyExo with Lipofectamine Messenger Max, and loading efficiency was quantified by Qubit assay. Cellular uptake was assessed by treating human chondrocytes with fluorescently labeled Exos or Cationic HyExos for 4 h, followed by confocal imaging. To evaluate cartilage penetration, 6 mm × 1 mm half-discs of 50% GAG-depleted cartilage were mounted in a custom transport chamber with particles applied to the superficial zone and PBS in the downstream chamber; after 24 h at 37 °C, explants were sectioned and imaged by confocal microscopy. Functional mRNA delivery was assessed by treating chondrocytes with eGFP-loaded Exos or Cationic HyExos and monitoring eGFP expression 18 h post-transfection. Bioactivity of IVT IL-1Ra mRNA delivery was confirmed by treating human chondrocyte micromass cultures for 18 h and quantifying IL-1Ra secretion in the culture medium by ELISA.

RESULTS: Hybrid Exos were generated by extrusion of Exos with Lipos. Hybrid Exos maintained a size comparable to native Exos while displaying intermediate surface charge characteristics (**Fig 1B**). HybridExos achieved a ~3-fold increase in mRNA loading compared to Exos (**Fig. 1B**). Co-extrusion with cationic Lipos was used to synthesize Cationic HyExos. These Cationic HyExos retained Exo-like size, exhibited charge reversal from –19.7 ± 1.3 mV to –1.1 ± 1.2 mV, and showed a ~3-fold increase in mRNA loading capacity (**Fig. 1B**). TEM imaging confirmed spherical morphology (**Fig. 1C-i**), and dual labeling demonstrated successful Exo–Lipo membrane fusion (**Fig. 1C-ii**). Compared with Exos, Cationic HyExos exhibited higher uptake by human chondrocytes in monolayer culture (**Fig. 1D**) and enhanced transport across 50% GAG-depleted cartilage, attributed to electrostatic interactions (**Fig. 1E**). Functionally, Cationic HyExos achieved greater eGFP expression than Exos and comparable expression to Lipofectamine (**Fig. 1F**). Delivery of IL-1Ra mRNA by Cationic HyExos further resulted in elevated IL-1Ra protein secretion at 18 h post-transfection, confirming the bioactivity of the delivered transcript (**Fig. 1G**).

DISCUSSION: We engineered Cationic HyExos that combine high gene-loading capacity with cartilage-targeting and penetration. These hybrids effectively delivered eGFP and IL-1Ra mRNA to human chondrocytes **demonstrating their potential as a non-viral platform for OA gene therapy**. Ongoing studies are investigating the disease-modifying effects of IL-1Ra mRNA delivery *in vitro* and *in vivo*.

SIGNIFICANCE: Cationic hybrid exosomes enable efficient mRNA delivery, targeted cartilage penetration, and protein expression in chondrocytes, offering a promising non-viral platform for disease-modifying gene therapy in osteoarthritis.

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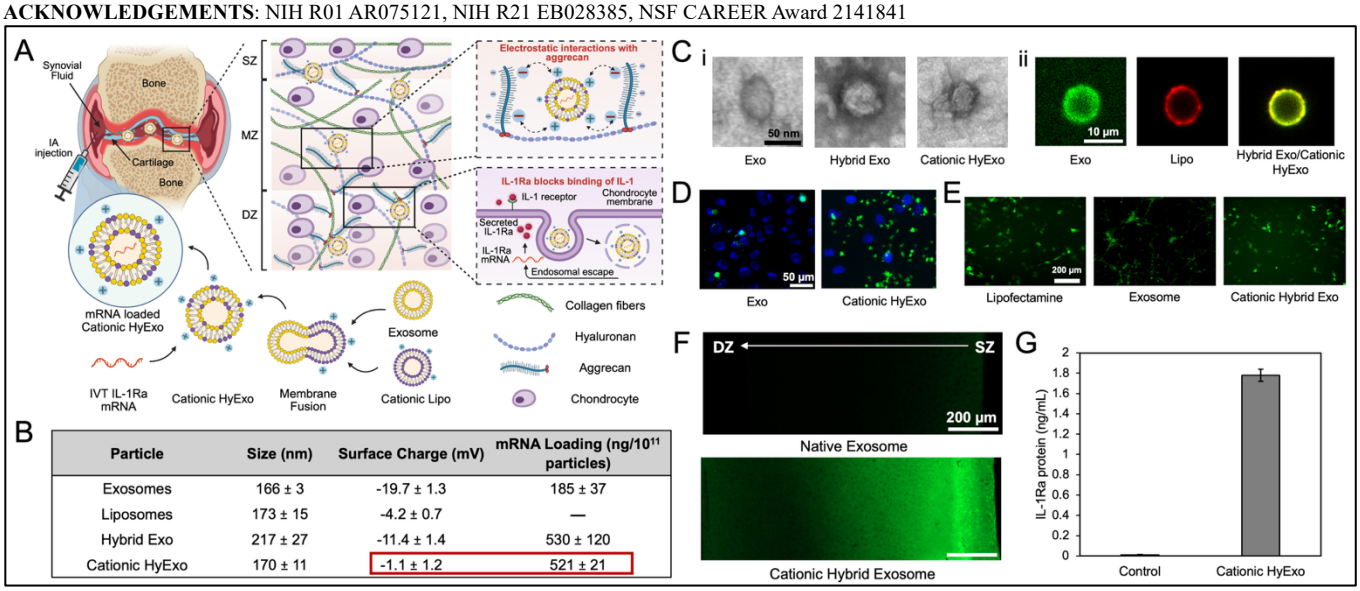

Particle	Size (nm)	Surface Charge (mV)	mRNA Loading (ng/10^{11} particles)
Exosomes	166 ± 3	-19.7 ± 1.3	185 ± 37
Liposomes	173 ± 15	-4.2 ± 0.7	—
Hybrid Exo	217 ± 27	-11.4 ± 1.4	530 ± 120
Cationic HyExo	170 ± 11	-1.1 ± 1.2	521 ± 21

Fig. 1: A) Schematic representing electrostatic based delivery of IVT IL-1Ra mRNA to chondrocytes in deep layers of cartilage by cationic hybrid Exos. **B)** Size, surface charge, and mRNA loading of Exos and hybrid Exos. **C) (i)** TEM imaging of hybrid Exos and **(ii)** dual labeling confirmation of cationic hybrid Exo. **D)** Chondrocyte uptake of cationic hybrid Exo. **E)** Transport properties of cationic hybrid Exo in 50% GAG depleted cartilage explants. **F)** *in vitro* eGFP expression in human chondrocytes. **G)** IL-1Ra protein production in human chondrocytes. SZ: superficial zone; MZ: middle zone; DZ: deep zone.