Targeted Delivery of Silencing RNA Utilizing Nanofibrous Polyester Materials

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Statement of Purpose

Over 1.1 million stents and 70,000 prosthetic grafts are implanted annually in the United States, with failure of these devices due to cellular occlusion still persisting. Unregulated cellular growth within the body can have catastrophic consequences as evidenced in cardiovascular disease where abnormal proliferation of neointimal smooth muscle cells is central to lesions of atherosclerosis and restenosis.

The goal of this study was to synthesize and characterize *in vitro* novel nanofibrous materials that will locally release silencing RNA (siRNA) directly to the implant site of medical devices such as stents and artificial blood vessels. Our hypothesis is that siRNA can be incorporated into nanofibers of the resulting materials using our proprietary technology and subsequently released in a slow, sustained fashion, thereby targeting localized cellular signaling directly around the implant. We employed electrospinning technology in order to synthesize the nanofibrous polyester materials. A major benefit of this process is that the polyester nanofibers are formed at low temperatures, unlike standard polyester fibers which are extruded as a melt at high temperatures. The low temperature permits the structure of the active compound to remain intact, thus retaining biological activity. Additionally, no exogenous binder agents or polymers are required to incorporate the respective agent.

Methods

Electrospinning Methodology: A DyLight 547-labelled siRNA determined to inhibit intimal hyperplasia (siMARCKS*) was incorporated into nanofibrous polyester fibers via electrospinning technology. Two concentrations of siRNA* (1.5X and 3.0X) were incorporated into the electrospun nanofibrous polyester materials (nPET-siMARCKS*-1.5X and nPET-siMARCKS*-3.0X, respectively). A nanofibrous polyester material without any siMARCKS* was also electrospun and served as the control material (nPET Control). Tubular (4mm internal diameter) and flat sheet (8cm X 10cm) constructs were electrospun.

Material Characterization: Control and siMARCKS*-loaded tubular and flat constructs were randomly selected and examined via a SEM (15kV accelerating voltage, 500X and 2,500X magnification; gold sputter coated for 60 seconds) in order to qualitatively assess fiber size and distribution throughout the materials. For fiber microscopy, random sections (1.5cm length X 1-2mm width) of the respective materials were selected and placed into a fiber microtome. Sections (1μm thickness) were then cut, placed onto a slide, observed using visible light (100X) and a TRITC-filter (100X) and photographed using a digital camera.

Determination of Physical Properties: Control and siMARCKS*-loaded nPET materials (5mm width, 2cm length; n=2/test condition) were measured and cut. A Q-Test Tensile Strength Apparatus was calibrated according to manufacturer's specifications in a climate-controlled environment. Segment stretching (crosshead speed = 50mm/min, gauge length = 1cm, load cell = 25 lb) was then initiated and terminated upon segment breakage. For suture retention, a 24-gauge wire was passed (one pass) through one wall on the end (3mm from edge) of a 1.5cm length tubular segment of control and siMARCKS-loaded nPET materials. The wire and opposite end of the conduit was then attached to the Q-Test apparatus, respectively (approximate clamp distance – 2.6cm). The force (grams) required to pull the wire through the control and siMARCKS*-loaded nPET materials was determined using a pull rate of 5cm/min.

In Vitro Wash Studies: nPET control and siMARCKS*-loaded nPET flat materials were evaluated for siMARCKS* release upon exposure to PBS under static flow conditions. Flat materials from each group were cut into 1cm^2 pieces (n = 6 segments/test condition) using a Texas Custom Die. Segments were then weighed and measured for thickness using an Ames Thickness gauge. Initial surface fluorescence for all control and test segments was measured by spectrofluorometry. To each segment, 1ml of PBS (at 37°C) was added. Tubes with materials/PBS were then capped and placed into a 37°C water bath. At time periods of 1, 24, 48, 72, 96, 120, 144, 168 and 192 hours, samples were removed from their respective tubes, blotted in Kimwipes to removes excess PBS and surface fluorescence read. After assessing surface fluorescence, each wash solution was evaluated for fluorescence in order to determine if siMARCKS* release could be detected in the solution (1ml solutions of PBS (n = 3) served as the blank).

siMARCKS* Uptake from nPET Materials: For transfection from materials, nPET control and nPET-siMARCKS* materials were cut into 16mm diameter circles and placed in custom designed seeding chambers. Human coronary artery smooth muscle cells (HCASMCs; 25,000 cells/well) were then added to each well in complete media (no siMARCKS* added). Cells were incubated on the materials for a total of 3 days with one complete change to fresh media (no siMARCKS added) on Day 1 post-plating. HCASMCs exposed to siMARCKS* which was added directly into the tissue culture well served as a positive transfection controls for these experiments. On Day 3, cell nuclei were stained via Hoechst 33342. Cells grown directly on well surfaces, with fluorescent siMARCKS*, were directly assessed for transfection by viewing *in situ* with the fluorescence microscope. Cells from all groups were exposed to 1X "TrypLE Express" trypsin substitute for 30 minutes in a 37°C incubator, followed by concentration of the released cells via centrifugation and resuspension. These suspensions were examined by light/ fluorescence microscopy.

Results/Discussion

nPET tubular and flat constructs with fluorescently-labelled siMARCKS* had a pinkish hue as compared to nPET control materials. This hue increased in intensity between the 1.5X and 3.0X materials. Average wall thickness for several nPET control (0.48 $\pm$ 0.17mm; n = 6 segments), nPET-siMARCKS*-1.5X (0.45 $\pm$ 0.13mm; n = 6 segments) and nPET-siMARCKS*-3.0X (0.44 $\pm$ 0.09mm; n = 6 segments) lots were comparable.

SEM assessment revealed that both control and siMARCKS*-loaded nPET materials had a blend of sub-micron to nanometer sized fibers. Increasing siMARCKS* concentration in the polymer did not seem to alter fiber morphology as compared to the nPET control. Fiber microscopy revealed that there was minimal background fluorescence from the control segment. In contrast, significant fluorescence was present for both nPET-siMARCKS* materials.

Tensile strength of the nPET-siMARCKS* materials (0.240 $\pm$ 0.004lb/mg and 0.213 $\pm$ 0.006lb/mg, respectively) was greater than the nPET control (0.122 $\pm$ 0.012lb/mg) construct. Suture retention was comparable between the nPET-siMARCKS* materials (480 $\pm$ 54g (1.5X) and 401$\pm$ 79g (3.0X), respectively) and nPET control materials (326 $\pm$ 115g).

siMARCKS* uptake from solution was confirmed with bright clear signal using the TRITC filter. Despite low cell yields off of all nPET-PBT materials, HCASMCs had siMARCKS* uptake from both the 1.5X and 3.0X materials as compared to cells from the nPET control materials, which had no fluorescence.

Conclusions

This type of siRNA delivery system would be one of the first systems to directly employ the device surface to locally deliver siRNA without the use of any exogenous binder or transfection agents, thereby improving patient morbidity and mortality.