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Enhanced gene delivery in porcine vasculature tissue following incorporation of adeno-associated virus nanoparticles into porous silicon microparticles

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ABSTRACT

There is an unmet clinical need to increase lung transplant successes, patient satisfaction and to improve mortality rates. We offer the development of a nanovector-based solution that will reduce the incidence of lung ischemic reperfusion injury (IRI) leading to graft organ failure through the successful *ex vivo* treatment of the lung prior to transplantation. The innovation is in the integrated application of our novel porous silicon (pSi) microparticles carrying adeno-associated virus (AAV) nanoparticles, and the use of our *ex vivo* lung perfusion/ventilation system for the modulation of pro-inflammatory cytokines initiated by ischemic pulmonary conditions prior to organ transplant that often lead to complications. Gene delivery of anti-inflammatory agents to combat the inflammatory cascade may be a promising approach to prevent IRI following lung transplantation. The rationale for the device is that the microparticle will deliver a large payload of virus to cells and serve to protect the AAV from immune recognition. The microparticle–nanoparticle hybrid device was tested both in vitro on cell monolayers and *ex vivo* using either porcine venous tissue or a pig lung transplantation model, which recapitulates pulmonary IRI that occurs clinically post-transplantation. Remarkably, loading AAV vectors into pSi microparticles increases gene delivery to otherwise non-permissive endothelial cells.

1. Introduction

Acute lung injury (ALI) and its most severe manifestation, acute respiratory distress syndrome (ARDS), are clinical syndromes defined by acute hypoxemic respiratory failure and bilateral pulmonary infiltrates consistent with edema. In-patient mortality is 38.5% for ALI, and 41.1% for ARDS [1]. Common causes of ALI/ARDS are sepsis, trauma, multiple blood transfusion, aspiration, and injury from toxic inhalation [1–3]. At this time, low tidal volume ventilation remains the only intervention that affords mortality benefit. Therefore, clinical approaches based on interruption of pathways identified in the acute phase are of interest for the development of strategies that can significantly lower the current mortality rates. Typically, a patient's intrapulmonary inflammatory response begins prior to the onset of clinically defined ALI and is most intense in the first 3 days after the onset of ALI/ARDS.

Unlike most animal models for ALI that rely on the administration of an insulting agent (toxins, O2, bacteria, etc.), we have developed a pig lung transplantation model that is the reproduction of the lung ischemia/reperfusion injury (IRI) that occurs clinically post-surgical transplantation. A hybrid nanotechnology platform has been created for the sustained expression of anti-inflammatory agents to combat the inflammatory cascade. A "Trojan horse" [2,3] mesoporous silicon (pSi) microparticle is envisioned to escort its nanovector payload to inflammation-associated endothelium, where it is internalized and subsequently releases the therapeutic nanoparticles [4,5]. Proven silicon fabrication processes allow exquisite control over particle geometry (dimensions from 100 nm and above) and porosity (pore size range between 5–100 nm) to create particles with specific size, shape, and surface treatments optimized for specific cargo and surface functionalization [6,7].

Due to their efficient role as gene delivery vectors, a number of viruses are currently being explored for therapeutic gene expression [8]. Because of its lack of pathogenicity, adeno-associated virus (AAV) is one of the most commonly investigated viral vectors. AAV can be used to deliver genes or RNAi [9]. Simple in structure, this virus is composed of a 25 nm protein capsid harboring a 4.7 kB single-stranded DNA genome.

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AAV is naturally present in a variety of hosts, including humans, but not pathogenic. To date, at least 12 serotypes have been isolated [10], with the most commonly used serotype for gene therapy being AAV2. Approximately 80% of the population has been exposed to this serotype [11].

We hypothesize that our "Trojan Horse" design concept will provide several important benefits to the immunotherapy approach. First, and the focus of this study, the pSi platform will enable the delivery of large payloads of VNPs into the endothelium, effectively increasing the local dosage of therapeutic genes in the target cells resulting in improved efficacy. Second, the pSi microparticle will shield VNPs from host anti-virus responses, such as neutralization by preexisting antibodies. Furthermore, the microparticle provides a facile means of combining multiple types of VNPs, each carrying a different transgene, and ensuring that combinations of genes get delivered to the same target cell. Additionally, other bioactive moieties can be incorporated into the microdevice, further enhancing the immunomodulatory capabilities of the vector. Lastly, the pSi microparticle can be designed to allow for controlled release of VNPs, consequently resulting in the sustained expression of anti-inflammatory factors.

Integrating the use of normothermic *ex vivo* circulation in conjunction with our novel "Trojan Horse" approach presents an exciting potential technique to effectively reduce the risks associated with systemic delivery by bypassing the negative effects of filter organ clearance and by minimizing the interaction with circulating immune-competent cells. The *ex vivo* model provides the most promising setup: an isolated viable organ model with normal metabolic functions. The major drawback of all the present perfusion systems is that their use is based on the concept of preserving the organ at approximately 4 °C by using a cold flushing solution. The lowering of the cellular metabolic rate is necessary to maintain organ viability. This is an obvious impediment for the delivery of any agents to the allograft, as all the crucial phases depend on metabolic processes. Improved technologies, such as the development of improved pumps and improved ventilators and the development of a lung-specific perfusion solution (Steen solution), which has an optimized composition and oncotic pressure, led to the rebirth of the notion of *ex vivo* perfusion. The lung has some very peculiar characteristics, as it consists mainly of elastic tissue with a low metabolic requirement. Normally the alveolo-capillary membrane receives its nutrition mostly by direct diffusion and it is possible to sustain the lung tissue by delivering oxygen through the trachea, via mechanical ventilation. Therefore, there is no need to perfuse with a cold solution to lower the metabolic rate. The possibility of treating an entire isolated organ, while circulating a normothermic solution and oxygenating through an endotracheal tube creates the ideal milieu for effectively delivering therapeutic agents *ex vivo* to address potential complications associated with lung transplant such as IRI.

In this study, we describe optimization of AAV2 loading into pSi microparticles and cell type specific expression of genes delivered by free or encapsulated AAV2. Delivery and expression of the pSi-AAV-GFP vectors *ex vivo* in vascular endothelium is presented as well as expression of the reporter gene in porcine pulmonary tissue following *ex vivo* vascular perfusion of the pSi-AAV2-GFP.

2. Materials and methods

2.1. Virus production

AAV2-GFP was produced using HEK293T cells and the triple transfection method [12]. Plasmids pXX6, pXX2, and pAAV-GFP were transfected into cells using polyethyleneimine (PEI). Virus was harvested 72 h later and purified using density gradient ultracentrifugation. Further purification was performed using a heparin affinity column (GE Healthcare) followed by dialysis into DPBS (+Mg/Ca). Virus concentration was quantified using QPCR and primers against the CMV promoter contained within the DNA cassette and standards bearing the CMV promoter. Viral capsids were dissolved with 2 M NaOH at 56 °C for 30 min and then neutralized with 2 M HCl.

2.2. Silicon microparticle functionalization

Oxidized porous silicon discoidal microparticles with dimensions of 1000 nm × 400 nm (pore size ~50–60 nm) were centrifuged to remove isopropanol and vacuum desiccated. Particles were functionalized with $-NH_2$ (3-aminopropyl-triethoxysilane), $-CH_3$ (methyltrimethoxysilane), –COOH (2-(carbomethoxy) ethyltrimethoxysilane), or –PEI (trimethoxysilylpropyl modified (polyethylenimine)), obtained from Gelest. Dry particles were resuspended in silanes diluted to 2% in 95% *v/v* isopropanol in water. Functionalization occurred for 2 h at 35 °C with 1300 RPM shaking. Particles were then rinsed three times with fresh isopropanol and vacuum desiccated in preparation of loading. The surface charge of each particle type was determined by suspending the particles in phosphate buffer and using a Malvern Zetasizer to determine zeta potential.

2.3. Loading AAV2-GFP within functionalized particles

Microparticles were dried (2e8 particles) and then resuspended in AAV2-GFP in DPBS (50 μl, ~1e12 vector genomes/ml). Loading was allowed to occur for either 15 or 30 min at room temperature. After this time particles were pelleted, rinsed twice with sterile water (100 μl each rinse), and then resuspended in 50 μl water. Loading was determined using QPCR. The same procedure was used as above, as 2 M NaOH dissolves silicon microparticles in addition to virus capsids. The presence of dissolved particles was found to not interfere with the PCR reaction (data not shown).

2.4. Nanoscale evaluation of pSi surface modification and VNP loading

Atomic force microscopy (AFM) was used to image surface topography of oxidized or silane-modified (2% APTES; 2 h incubation) pSi microparticles. Height and peak force measurements of particles were made using a Bruker Dimension ICON AFM. PeakForce tapping was performed using ScanAsyst to adjust imaging parameters and a model ScanAsyst Air probe made of silicon nitride with a silicon tip, and a spring constant of 0.4 N/m, 1 Hz scan rate. Analysis of AAV adhesion to a silicon wafer was performed using a Bruker Multimode AFM.

2.5. Cell culture

Gene delivery efficiency was determined in three cell lines: HeLa, HEK293T, and HMVEC (human microvascular endothelial cells). All cells were cultured at 37 °C with 5% CO_2. HeLa and HEK293T cells were maintained in DMEM with 10% FBS and 1% penicillin/streptomycin. HMVECs were cultured in EGM (Lonza).

2.6. Determination of gene delivery using flow cytometry

Cells were plated in 24 well plates such that they would be 75% confluent after 24 h. Transduction studies were performed by removing media and replacing with 220 μl fresh media. Either free AAV2-GFP or particle-loaded AAV2-GFP (each surface chemistry) was added to the media at a virus MOI of 1,000. After 3 h the media was removed and replaced with 1 ml fresh media. Cells were then cultured for 3 days before harvesting for flow cytometry analysis of GFP expression. Analysis was performed using a BD Fortessa using the FITC channel to detect GFP expression.

2.7. Confocal imaging of gene expression

HEK293T cells were seeded in 8 well chamber slides (BD) such that they would be 75% confluent after 24 h. Media was removed and

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