Personalized nanomedicine for the treatment of vascular hypertension in polycystic kidney disease models

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ABSTRACT

In our current study, we designed a nanomedical device for the treatment of vascular hypertension in polycystic kidney disease (PKD) model through cilia targeting. We generated and compared two different metal and polymer cilia-targeted nanoparticle drug delivery systems (DDS), i.e. gold (Au) and poly-actic-co-glycolic acid (PLGA) nanoparticles (NPs). These DDS were targeted to dopamine-receptor type-5 (DR5) on primary cilia and also loaded with fenoldopam (FD). The structures and sizes of the DDS were visualized with transmission electron microscopy (TEM). The size of the DDS was also confirmed with the dynamic light scattering. The diameters of Au-NPs and PLGA-NPs were approximately 40±2.5 and 102±4.8 nm, respectively. The surface charge of Au-NPs (−47.3 ± 1.2 mV) was significantly more negative than PLGA-NPs (−25.9 ± 1.0 mV). Fourier transform infrared spectroscopy (FTIR) also confirmed the conjugation of DR5 antibody with both DDS. Our *in vivo* studies reveal that both DDS showed improved blood pressure in PKD mice through NO mediated vasodilation.

Keywords: nanomedicine, ciliopathy, hypertension, primary cilia, chemosensory, imaging.

1 INTRODUCTION

Primary cilia are cellular organelles that function as sensory compartments [1]. Dysfunction in the chemosensory or mechanosensory of primary cilia results in a list of clinical diseases (ciliopathies) include expanding spectrum of kidney, liver, and heart disorders [2,3]. There is currently no treatment available for patients with cilia dysfunction. Nanoscience promises huge clinical impacts on disease management and personalized medicine. Nanoparticles have been used for targeted drug delivery at a desired site and a sustained drug release [4,5]. As chemosensory organelles, cilia have various receptors that can be activated by small molecules and hormones. There are multiple G-protein-coupled receptors that have been localized to cilia including serotonin, somatostatin, melanin, and dopamine receptors [6,7]. Because cilia are tiny cell organelles with a diameter of ~250 nm, we used DAu and PLGA-NPs as our drug delivery systems due to its excellent biocompatibility, stability properties and applicability for use *in vivo* to target primary cilia [8].

2 MATERIALS & METHODS

Unless otherwise indicated, most of the chemicals used in the present study were of high purity and purchased from the Sigma Aldrich Inc., USA. Mice were housed in an animal facility at the University of California Irvine (UCI) under federal, state and national institute of health guidelines and approved by the Animal Care and Use Committee Guidelines of UCI.

2.1 Synthesis of nanoparticles

DAu and PLGA nanoparticles were synthesized as simple reduction and single emulsion method, respectively [8]. The DR5 antibody was generated from a synthetic peptide corresponding to amino acids 2-10 of the DR5 N-terminus, and it did not cross-react with other dopamine receptors. Initially, we conjugated DR5 to AlexaFlour 594 maleimide using an AlexaFluor 594 antibody labelling kit to target thiol groups, according to manufacturer's instructions (Thermo Fisher Scientific, MA). The pre-conjugated DR5-AlexaFluor 594 antibody and fenoldopam were bound to the synthesized Sunbright-40-OA-DDS using a previously reported method [9]. Briefly, Sunbright-40-OA-DDS (100 mg) were cooled to 4 °C, mixed with 500 µg of DR5-AlexaFluor 594 antibodies to a final volume of 25 mL in PBS and shaken overnight at 4 °C. A DMSO solution of fenoldopam (400 µL, 15 mg/mL in each reaction) was added to the NP solution, and the reaction was allowed to proceed under stirring (400 rpm) for another 16 hours at 4 °C. The antibody- and fenoldopam-loaded Sunbright-40-OA-DDS were separated from the free antibody and free drug. The cilia targeted DDS were then washed with PBS several times, lyophilized and stored in the dark.

2.2 Nanoparticle characterization

For measurements of size and shape, the synthesized nanomaterials were examined by TEM using an FEI/Philips 200 kV CM-20 electron microscope. Formulated cilia targeted DDS were freeze-dried and the dried powder was used for X-ray diffraction using a Rigaku SmartLab X-ray diffractometer and Cu-Kα (Cu target) radiation at a scanning rate of 1° per min in the region of 2θ=10-90°. X-ray photoelectron spectra of the samples were recorded on a Kratos Analytical AXIS Supra system with a monochromatic Al/Ag X-ray source (Al target). The particle size distribution and zeta potential value of the synthesized nanomaterials was analyzed using a Malvern Instruments Zetasizer Nano Series-ZS-90. The FTIR spectra were recorded using Bruker spectrometer in the diffuse reflectance mode at a resolution of 4 cm^{-1}.

2.3 Cell culture

Epithelial cells were purchased (ATCC) and cultured in Dulbecco's Modified Eagle Medium (Corning Cellgro, NY) supplemented with 10% fetal bovine serum (HyClone, MA), and 1% penicillin-streptomycin (Corning Cellgro, NY) at 37 °C in a humidified, 5% CO_2 environment. Prior to the experiments, antibiotics were withdrawn, and cells were serum starved for 24 hours to induce differentiation.

2.4 Immunocytochemistry

For the *in vitro* cilia length measurements, epithelial cells were grown on the formvar membrane. Primary cilia consisting of acetylated microtubule structures were measured by direct immunofluorescence staining with an acetylated-α-tubulin antibody following a 16 hours incubation with different concentrations (0.1-5 µg/mL) of cilia targeted DDS. Likewise, the cilia targeted DDS without loaded FD were used as the corresponding control. FD alone was also used as another control. Cells were rinsed with buffer (sodium cacodylate), fixed with 2.5% glutaraldehyde in 0.2 M buffer for 10 min, and permeabilized with 1% Triton-X 100 in buffer for 5 minutes. An antibody against acetylated-α-tubulin and the secondary antibodies were also diluted in 10% FBS to decrease the background florescence; a FITC-conjugated secondary antibody.

2.5 Intracellular Ca^{2+} and NO imaging

Cells were grown as monolayer on glass-bottomed plates to enable live microscopy imaging. After a 16-hour incubation without or with different concentrations (0.1-5 µg/mL) of FD or cilia targeted DDS, cells were loaded with 5 µM Fura2-AM at 37 °C for 30 minutes. After washing to remove excess Fura-2 AM, cytosolic calcium (Ca^{2+}) images were captured every second by recording the fluorescence of Ca^{2+}-bound Fura-2 AM at an excitation wavelength of 340/380 nm and an emission wavelength of 510 nm.

For intracellular nitric oxide (NO) measurements, cells were loaded with 20 µM DAF-FM for 30 minutes at 37 °C. NO was then measured every second at excitation and emission wavelengths of 495 and 515 nm, respectively. Fluid shear stress was then applied to cells through InsTech P720 peristaltic pumps with an inlet and outlet setup. The fluid was perfused through cell monolayers at a sub-minimal shear stress of 0.5 dyn/cm^2.

2.6 Animals

One-week-old *Tie2Cre•Pkd2*$^{WT/WT}$ (with Cre activation; control group), *Tie2Cre•Pkd2*$^{flox/flox}$ (without Cre activation; control group) or *Tie2Cre•Pkd2*$^{flox/flox}$ (with Cre activation; experimental group) mice were intraperitoneally injected with 250 µg of tamoxifen in a 50-µL volume daily for five consecutive days. Mice were treated with cilia targeted DAu/PLGA-NPs every 72 hours for 8 weeks. On the other hand, FD alone was perfused for 30 minutes every 72 hours for 8 weeks.

2.7 Mouse blood pressure measurements

Tie2Cre•Pkd2$^{WT/WT}$, *Tie2Cre•Pkd2*$^{flox/flox}$ and *Tie2Cre•IFT88*$^{flox/flox}$ mice were subjected to blood pressure examining by the non-invasive tail-cuff method using a CODA high-throughput system (Kent Scientific, CT). Blood pressure was measured twice daily for the duration of the study after the initial three days of acclimating each mouse to the tail cuff. All measurements were performed by operators in a double-blind manner.

2.8 Statistical analysis

All quantifiable data are reported as the mean±standard error of the mean (SEM). The homogeneity of variance was verified within each data set. When a data set was not normally distributed or heterogeneous variance was detected, the distributions were normalized via log transformation. This approach produced normally distributed data sets. Statistical analysis was performed using ANOVA followed by followed by the Tukey post hoc test. The difference between groups was considered significant at $p < 0.05$.

3 RESULTS & DISCUSSION

We prepared stable DAu-NPs and PLGA-NPs using several synthesis steps. We analysed and characterized the materials from each synthesis and surface functionalization i.e. bare NPs to functional cilia-targeted nanoparticle drug delivery systems (DDS). The structures and sizes of cilia targeted DDS were visualized with electron micrographs (**Figure 1**). The size of the cilia targeted DDS was also confirmed with the dynamic light scattering and are comparable with our TEM results. The diameters of cilia

targeted DAu-NPs and PLGA-NPs were 40±2.5 and 102±4.8 nm, respectively.

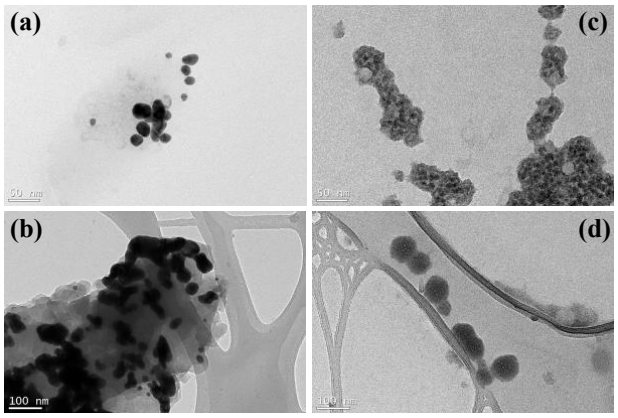

Figure 1. a) Transmission Electron Microscopy (TEM) images of a) DAu-NPs b) Surface functionalized cilia targeted DAu-NPs c) bare PLGA-NPs and d) core-shell cilia targeted PLGA-NPs.

The time-dependent cilia length increase by DDS was analyzed in various cell types. In these studies, we consistently observed that 16 hours of treatment was an optimal effect of DDS on primary cilia. After 16 hours, the efficacies of DDS were thus examined and compared to their respective control (PBS treatment) (**Figure 2**). FD-alone was also used as a positive control. FD-loaded DDS and FD-alone significantly increased cilia length compared to their corresponding control, and there was no significant difference in cilia length between fenoldopam-alone and fenoldopam-loaded DDS [8].

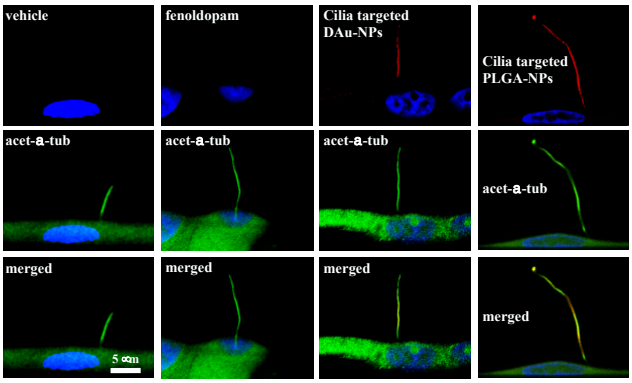

Figure 2. Fluorescence images show that fenoldopam and the DDS (red) increased the cilia length (16 h of treatment) compared with controls (vehicle; PBS treatments). The ciliary marker acetylated-α-tubulin (green) and a nuclear marker, DAPI, (blue) were used (adapted from [8]).

Primary cilia function has been primarily examined by monitoring fluxes in cytosolic Ca^{2+} concentrations. Therefore, the cytosolic Ca^{2+} indicator Fura-2AM was used to differentiate cilia function by perfusion fluid flow. Because an increase in cilia length translates to an increase in cilia function, shear stress was reduced from 1.0 to 0.5 dyn/cm^2 to magnify changes in sensitivity in terms of Ca^{2+} signalling in control cells compared with that in the DDS-treated cells. As expected, fluid-flow shear stress induced an increase in the cytosolic Ca^{2+} concentration (**Figure 3**) [8].

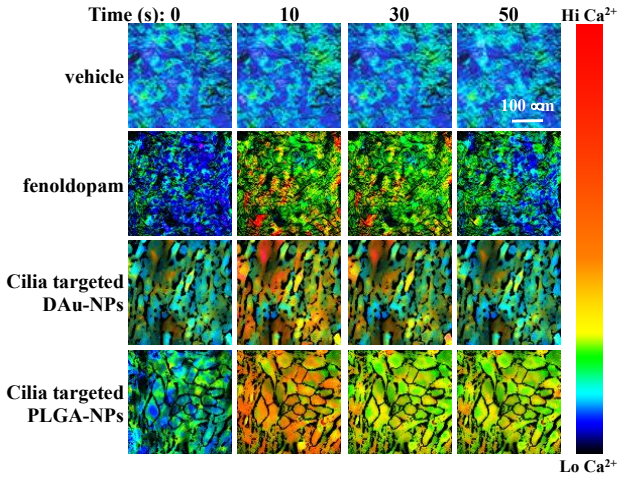

Figure 3. Cytosolic Ca^{2+} was visualized with the Ca^{2+}-sensitive fluorescent dye Fura-2-AM. Ratiometric images from 340 and 380 nm excitation wavelengths were captured. Numbers on the top of the representative images display the time in seconds (s). A sub-minimal fluid shear of 0.5 dyn/cm^2 was applied to the cells to induce Ca^{2+} flux. The pseudocolor indicates Ca^{2+} levels. The color bar indicates the levels of Ca^{2+} (adapted from [8]).

Although renal epithelial cells synthesize and release NO, researchers have not determined whether cilia are involved in this process. The treatment of epithelial cells with the DDS was sufficient to evoke a sustained release of NO (**Figure 4**). Fluid flow-induced cilia bending can activate intracellular Ca^{2+} followed with NO biosynthesis, which are used as indices to measure cilia function. While FD-alone and FD-loaded DDS significantly increased cytosolic Ca^{2+} (**Figure 3**) and NO biosynthesis (**Figure 4**) compared to their corresponding controls, there was no difference in cilia function among cell populations treated with FD-alone and FD-loaded DDS [8].

To compare the efficacies among DDS and FD-alone, we used endothelia-specific *Pkd2* knockout mouse model. While cilia targeted DAu-NPs significantly reduced blood pressure in hypertensive *Pkd2* mice, cilia targeted PLGA-NPs further decreased blood pressure toward the wild-type's level (**Figure 5**). Short 30 min infusions of FD showed no long-term effect, indicating an advantage of sustained-release of DDS.

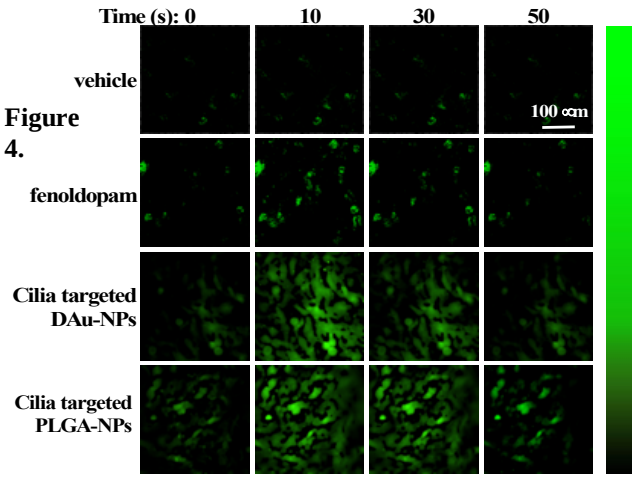

Figure 4. Intracellular NO synthesis was visualized with the NO-sensitive fluorescent dye DAF-AM. Effects of sub-minimal fluid shear stress showed greater NO production in treated cells than in control cells. The color intensity indicates NO levels. Numbers on the top of the representative images display the time in seconds (s). A sub-minimal fluid shear of 0.5 dyn/cm^2 was applied to the cells to induce NO flux. The color bar indicates the levels of NO (adapted from [8]).

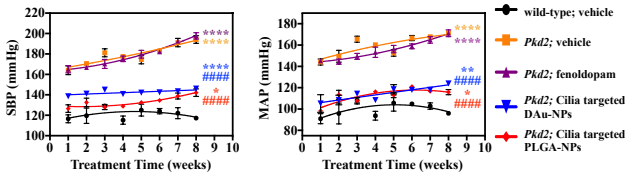

Figure 5. Blood pressure was measured for 8 weeks with different treatments. Systolic blood pressure (SBP) and mean arterial pressure (MAP) are shown in the line graphs (adapted from [8]).

While fenoldopam-alone seemed to have no effect on *Pkd2* mice, fenoldopam had an immediate effect during infusion. A short infusion of FD significantly decreased blood pressure followed by reflex tachycardia. While DDS did not show any advantages over FD -alone in cultured cells *in vitro*, DDS delivered FD more superior than FD-alone by eliminating the side effect of reflex tachycardia in PKD mouse model. Although slow infusion was required for fenoldopam-alone in mice, bolus injection was possible for DDS. Though there were no significant therapeutic differences between cilia targeted DAu-NPs and cilia targeted PLGA-NPs, cilia targeted PLGA-NPs tended to correct ciliopathy parameters closer to normal physiological levels, indicating cilia targeted PLGA-NPs were better cargos than cilia targeted DAu-NPs (**Figure 5**).

4 CONCLUSIONS

In summary, we introduced a novel approach to target primary cilia for the very first time. Also, our studies provided scientific evidence that existing pharmacological agent could be personalized with advanced nanomaterials to treat ciliopathy by targeting cilia without the need of generating new drugs. Our studies opened a paradigm of harnessing a novel mechanism for future strategies in nanomedicine toward a more personalized medicine for ciliopathy.

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