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RPPR Final Report
as of 13-Dec-2021

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Major Goals: The delivery of nucleic acids including siRNA, mRNA and CRISPR gene editing complexes represents a major challenge for the clinical translation of gene therapy. Polymer-, lipid nanoparticle- or virus-based platforms have been explored extensively but have not been able to achieve the safe and efficient delivery profiles. As an alternative strategy, we have developed techniques to design and synthesize DNA and RNA nanoparticles based on the DNA origami technique that provide unique opportunities for the loading of nucleic acid cargo. We are seeking to translate the delivery platform from an academic setting into commercial ventures. Key advantages of the nucleic acid-based nanoparticles include full synthetic control (1) over the chemical and structural composition of the delivery platform and (2) over functionalization valency, spacing and stoichiometry including the exterior attachment of targeting modalities for applications to cancer as well as genetic and infectious diseases and the interior attachment of cargo with (3) ideal synergy with siRNA, mRNA and CRISPR/Cas9 gene editing complexes. Major goals of this project are (1) the identification of key partners in the biotechnological industry and of promising therapeutic applications and (2) understanding the technical challenges of our nucleic acid-based nanoparticles and competing delivery platforms. This report will focus on these technical challenges in the context of pre-clinical development. Key questions include the immunotoxicity of nucleic acid-based nanoparticles and their biodistribution in animal models.

Accomplishments: During the reporting period, we have conducted several pilot mice studies to assess the immunotoxicity and biodistribution of DNA nanoparticles in vivo. Our key accomplishments are:

(1) Establishing the endotoxin-free production of wireframe DNA origami-based nanoparticles at scale for preclinical studies.

(2) Assessment of general toxicity parameters in mice after intravenous injection DNA nanoparticles. Monitoring the behavior, body weight and histology of key organs, we demonstrate that our delivery platform is non-toxic at typical therapeutic doses.

(3) Assessment of immunotoxicity in mice after intravenous injection of DNA nanoparticles. Cytokine profiling revealed that our delivery platform does not induce adverse innate immune responses at typical therapeutic doses.

(4) Biodistribution study in mice after intravenous injection of dye-labeled DNA nanoparticles. In vivo and ex vivo imaging revealed initial liver accumulation of our delivery platform followed by rapid renal clearance.

In summary, we conclude that our DNA nanoparticles constitute an immunologically inert, biodegradable delivery platform that with limited passive organ accumulation. We thus envision that our platform can be readily re-targeted for therapeutic applications.

Training Opportunities: Postdoctoral research associate Dr. Tyson Shepherd accepted an offer as application scientist with Inscripta, Inc.

Results Dissemination: Nothing to Report

Honors and Awards: Nothing to Report

Protocol Activity Status:

Technology Transfer: We have filed a provisional patent related to our DNA-based assembly technology for efficient gene therapy delivery detailed in this report. This patent is also included in the Products section of this report:

“Disclosed are compositions and methods involving nucleic acid assemblies that enclose and/or protect cargo. Disclosed are compositions that include a nucleic acid assembly comprising one or more nucleic acid molecules and cargo comprising two or more cargo molecules. The nucleic acid assembly can have physiochemical properties that: (i) enhance targeting of the composition to one or more types of cells, tissues, organs, or microenvironments relative to other types of cells, tissues, organs, or microenvironments in vivo; (ii) enhance stability and/or half-life of the composition in vivo; and/or (iii) reduce immunogenicity of the composition. The nucleic acid assembly and/or cargo can have features that enhance intracellular trafficking of nucleic acid assembly and/or its cargo. The cargo can be enclosed and/or protected by the nucleic acid assembly. Some or all of the cargo molecules in the composition can be present in a defined stoichiometric ratio.”

Feng Zhang, Tyson R. Shepherd, Rémi Veneziano, Mark Bathe, Ian Slaymaker and Bernd Zetsche: “Nucleic acid assemblies for use in targeted delivery” U.S. Patent Application No. US20210317479A1 – published 10/2021.

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Participant Type: Postdoctoral (scholar, fellow or other postdoctoral position)
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Project Contribution:
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RPPR Final Report
as of 13-Dec-2021

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Accomplishments

Motivation

Advances in human genome sequencing have identified a growing number of gene-specific mutations that result in rare genetic conditions causing disease in millions of people worldwide[1]. Clustered regularly interspaced short palindromic repeats (CRISPR) ribonucleoprotein complexes (RNPs) project to revolutionize the treatment of genetic diseases through genome editing via site-specific RNA-targeted DNA cleavage[2-5]. This targeted cleavage in cells initiates cellular DNA repair leading to either indel formation through non-homologous end joining (NHEJ) or gene correction through homology directed repair (HDR). Leveraging this gene editing system is promising, but similar to other gene therapy cargo including siRNA and mRNA, delivery of nucleic acids complexes is challenging and requires next-generation platforms to enable cell- and tissue-specific targeting and cellular uptake. Currently, CRISPR delivery is typically achieved by genetically encoding Cas protein expression using viral vectors or lipid nanoparticles which leads to off-target editing and genome instability[6]. Recombinantly produced, pre-assembled CRISP RNPs display reduced off-target editing but their in vivo delivery remains inefficient[6].

The Bathe laboratory has pioneered the algorithmic design of wireframe nanoparticles based on the DNA origami method[7-8]. The folding of long, single-stranded DNA (ssDNA) scaffolds by annealing of shorter ssDNA staples yields monodispersed nanoparticles of arbitrary geometry and size at the 10 to 100 nm scale. Using the programmability of Watson-Crick base pairing, these assemblies provide precise, independent control over the valency, spacing and stoichiometry of functionalization – for both nucleic acid cargo and targeting moieties. Here, we propose to use DNA-based (or RNA) nanoparticles (DNPs) to deliver CRISPR RNPs and other gene therapies. Specifically, DNA origami offers unique opportunities because of **(1)** the facile hybridization-based attachment of RNPs at controlled stoichiometry, **(2)** the co-formulation of HDR templates, **(3)** the potential for cell- and tissue-specific delivery using targeting moieties and **(4)** low toxicity profiles and full biodegradability. Towards the translation of our delivery platform, the Bathe laboratory and other have demonstrated the scalability of ssDNA scaffold and DNP production[9-10]. Building on these advances, we have focused on the pre-clinical characterization of wireframe DNA origami with regard to their baseline toxicity and biodistribution. Such characterization will establish the dosing regime, timescales and passive organ accumulation for prospective delivery studies.

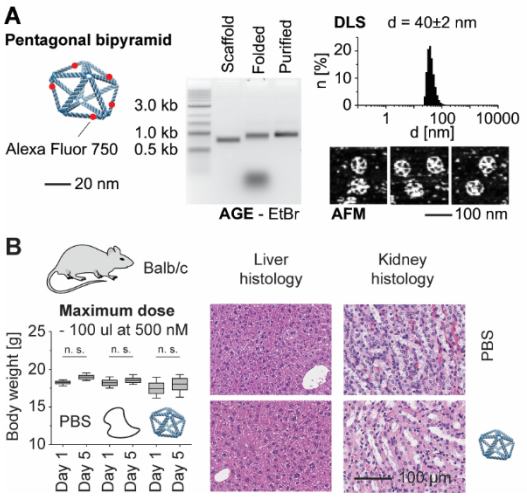

Figure 1. DNA origami synthesis and general toxicity studies. (A) We designed a pentagonal bipyramid DNP bearing 5 Alexa Fluor 750 dyes. The nanostructures were produced under endotoxin-free condition and characterized by AGE, DLS and AFM. **(B)** DNPs and ssDNA scaffold were injected intravenously into Balb/c mice. Monitoring of behavior and body weight indicated no toxicity increase at maximal doses of 50 pmol compared to the PBS control. No DNA-induced phenotype was observed by liver and kidney histology. (n = 5, Student's t-test, α = 0.05).

Accomplishment I – Assessment of toxicity

To assess the baseline toxicity of our delivery platform, we designed a pentagonal bipyramid using DAEDALUS with up to 5 functionalization sites for Alexa Fluor 750 dyes[11]. The required ssDNA scaffold was produced at scale using bacteriophages and subsequently purified from endotoxins. Assembled DNA origami was characterized using agarose gel

electrophoresis (AGE), dynamic light scattering (DLS) and atomic force microcopy (AFM) (**Figure 1A**). We initially evaluated the general toxicity profile of wireframe DNA origami after intravenous injection in mice compared to the administration of ssDNA scaffold and PBS (**Figure 1B**). Maximum doses of 50 pmol were chosen after consideration of production scale and maximum DNP concentrations to ensure monodispersity. Notably, the doses are comparable or higher to those used in gene therapy delivery studies using other classes of DNA origami[12]. Evaluation of toxicity by monitoring mouse behavior and body weight for 5 weeks post-injection, revealed no phenotype for DNPs or ssDNA scaffold compared to PBS. We further performed histological studies of liver and kidney – again without observing DNA-dependent phenotypes.

As exogeneous nucleic acids are recognized by the innate immune system, it is important to establish the immunotoxicity of DNPs and the systemic induction of cytokines. ssDNA scaffold and staples cover large sequence spaces and potentially contain CpG-like motifs that can induce TLR9-dependent signaling after endocytosis[13]. Exogenous, cytoplasmic DNA is recognized via the cGAS-STING pathway[14]. Accordingly, we investigated whether the intravenous injection of wireframe DNA origami at 50 pmol would induce typical DNA-dependent cytokine responses (**Figure 2**). Following blood draws at 3 and 24 h post-injection, IFN-α, IFN-γ, IL-6 and TNF-α profiles were monitored by ELISA. LPS was carried as a positive control. In accordance with the absence of general toxicity we observed no increase in cytokine profiles compared to injection of PBS. In summary, our results establish wireframe DNA origami as a non-toxic delivery platform at typical therapeutic, pre-clinical doses. This will pave the way to explore functionalized DNPs for the delivery of CRISPR RNPs and other gene therapy cargo.

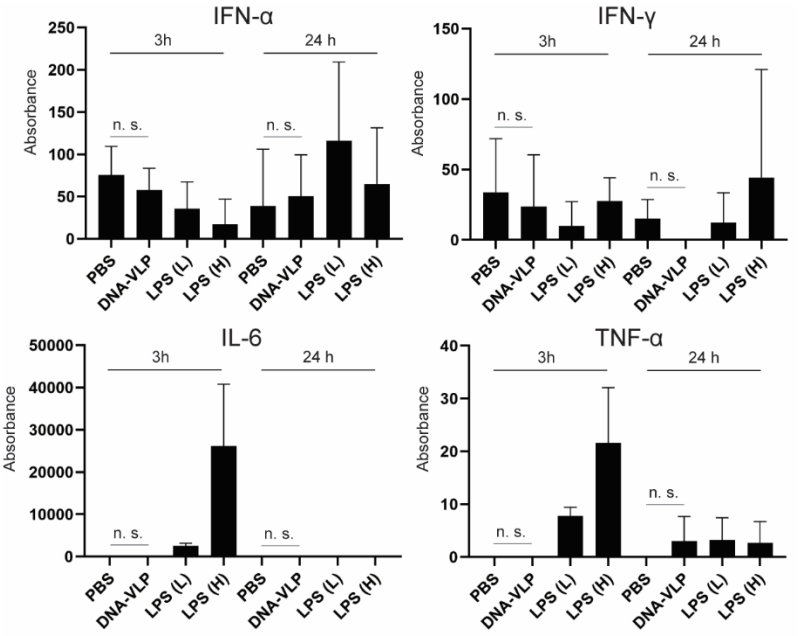

Figure 2. DNA origami immunotoxicity. 50 pmol of DNPs were injected intravenously into Balb/c mice. Monitoring of cytokines by ELISA for blood draws at 3 and 24 h post-injection did not reveal immunotoxicity. (n = 5, Student's t-test, one-sided, α = 0.05).

Accomplishment II – Biodistribution study

To expand the pre-clinical baseline characterization of wireframe DNA origami, we evaluated the biodistribution of our delivery platform. Balb/c mice were injected intravenously with 25 pmol of dye-labeled DNPs (**Figure 3A**). In vivo imaging revealed systemic distribution within the first 15 min followed by accumulation in the liver. Liver accumulation was detectable for up to 1 h after which we observed full renal clearance within 6 h. Liver accumulation for the dye-labeled ssDNA staple control was substantially reduced and renal clearance occurred within 2 h. Organ accumulation was further validated by ex vivo imaging, confirming distribution to the liver in addition to minor accumulation in spleen and kidneys. We suspect that renal clearance is partly driven by the nuclease-driven degradation of bare, non-protected DNPs[15]. Together with our

toxicity assessment, these results suggest that wireframe DNA origami projects to be safe and fully biodegradable in the context of pre-clinical studies. We observe only minor passive organ accumulation, facilitating the re-targeting of DNPs for specific therapeutic applications. Notwith-standing, initial experiments for the delivery of gene therapy cargo might target the liver, leverage passive DNP accumulation.

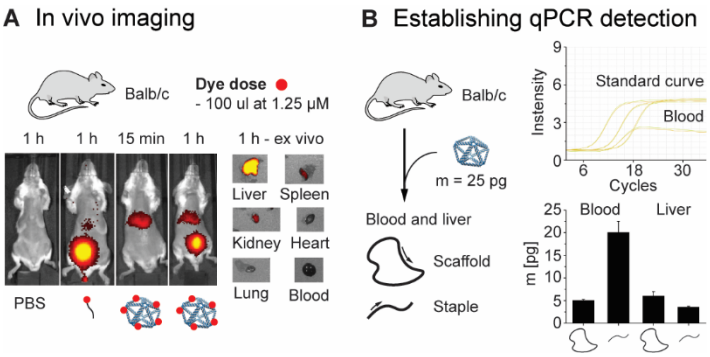

Figure 3. DNA origami biodistri-bution. (A) 25 pmol of dye-labeled DNPs were injected intravenously into Balb/c mice. In vivo imaging revealed initial liver accumulation that lasted for up to 1 h. After 6 h, full renal clearance was observed. Ex vivo imaging confirmed liver accumulation with minor accum-mulation inf spleen and kidneys. **(B)** We stablished the qPCR-based read-out of ssDNA scaffold and staples after spiking into blood and liver homogenate. This paves the way for multiplexed biodistribution studies with barcoded DNP libraries. (n = 3, representative images are shown).

Additionally, we established the qPCR-based detection of wireframe DNA origami in blood and liver in preliminary experiments. To this end, organ homogenates were spiked with 25 µg of DNP and primers were designed to independently detect ssDNA scaffold and staples (**Figure 3B**). These results pave the way for multiplexed biodistribution studies with barcoded DNP libraries that will include targeting moieties and additional wireframe geometries.

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