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TITLE: Extremophile RNA Delivery for Radioprotection in Prostate Cancer Patients

PRINCIPAL INVESTIGATOR: James Byrne, MD, PhD

CONTRACTING ORGANIZATION: Brigham and Women's Hospital

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Prostate cancer patients undergoing radiation therapy may experience severe debilitating short- and long-term toxicities resulting in reduced quality of life and regret of their treatment decisions. These toxicities are bystander effects based on proximity of normal organs to the treatment target and may manifest as urinary frequency, urinary obstruction, and rectal bleeding. Although there have been many attempts to mitigate these toxicities using radioprotectants, there are few clinically available radioprotectants. Newer methods to reduce the incidence of GU and GI side effects may provide substantial benefit to patients. Certain organisms in nature—known as tardigrades—have the ability to withstand extremely large doses of radiation as a result of a tardigrade-unique Dsup protein that prevents DNA damage. We propose the local delivery of mRNA for expression of the Dsup protein for radioprotection of mucosal surfaces. We hypothesize that inducing the expression of Dsup protein in normal tissues will impart a high degree of radioprotection.

15. SUBJECT TERMS
Tardigrades, mRNA delivery

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1. INTRODUCTION

Radiation therapy can cause significant short- and long-term toxicities in many prostate cancer patients. There are few clinically available radioprotectants for a variety of reasons, including off-target effects. Tardigrades have the ability to withstand extremely large doses of radiation as a result of a tardigrade-unique Dsup protein that prevents DNA damage. We proposed the local delivery of mRNA for expression of the tardigrade-specific damage suppressor (Dsup) protein for radioprotection of mucosal surfaces. We hypothesized that inducing the expression of Dsup protein in normal tissues will impart a high degree of radioprotection. To test this hypothesis, our goal was to encapsulate Dsup mRNA in poly(beta-amino esters) PBAE nanoparticles that can be applied to the urethra, bladder, and rectum.

2. KEYWORDS

Tardigrades, mRNA, damage suppressor protein, microneedles

3. ACCOMPLISHMENTS

Major Goals of this Project

	Timeline	Percent complete	Accomplished
Major Task 1: Formulate hydrogels and microneedles for delivery of Dsup mRNA	12 months	90%	
Major Task 2: Evaluate the safety of Dsup expression in a rat model	12 months (new proposed 18 months)	25%	
Major Task 3: Determine the kinetics of Dsup expression in the urethra, bladder, and rectum after hydrogel and microneedle delivery	18 months	0%	
Major Task 4: Quantify the level of radioprotection of the Dsup mRNA-PBAE loaded hydrogels and microneedles in rats and pigs	24 months	0%	

Accomplishments under these Goals

1. Major Activities: We had previously validated fabrication of microneedles and hydrogels with PBAE nanoparticles and confirmed in vitro transfection. Prior to transitioning these materials for in vivo application, we performed direct injection of PBAE nanoparticles into rectal and oral mucosa. There was no transfection with the PBAE nanoparticle system. As a result, we reformulated the nanoparticles to integrate lipids with the PBAE polymers, which demonstrated excellent in vitro in Caco-2 cells. This cell line was used in lieu of primary cells due to the ease of use and lifetime of these cell lines. In addition, we achieved successful in vivo transfection through direct injection in the oral mucosa in rats. Additional testing is underway to evaluate rectal administration through direct injection, as well as reformulating the nanoparticles in microneedles and gels for administration. Additional work is underway to formulate in microneedles.

2. Specific Objectives:

	Timeline	Percent completed	Accomplished
Major Task 1: Formulate hydrogels and microneedles for delivery of Dsup mRNA			
Subtask 1: Screen PBAE polymers for mRNA expression in vitro. *Milestone Achieved: Identification of top polymers for mRNA expression*	6 months	100%	X
Subtask 2: Fabricate mucosal hydrogels and microneedles out of rapidly dissolvable biocompatible polymers	6 months	100%	X
Subtask 3: Assess microneedle penetration in ex vivo tissue	6 months	100%	X
Subtask 4: Evaluate mRNA integrity in vitro post-processing. *Milestone Achieved: Generate clinically relevant formulations for mRNA delivery*	6 months	100%	X
Subtask 5: Validate in vitro expression of Dsup protein from microneedles and hydrogels	8 months	100%	X
Subtask 6: Evaluate stability of mRNA loaded into microneedles for up to 28 days. *Milestones Achieved: Multiple methods for delivery and expression of Dsup protein have been generated*	8 months	25%	X
Major Task 2: Evaluate the safety of Dsup expression in a rat model			
Subtask 1: Assess the acute mucosal reaction after hydrogel and microneedle administration in rats	12 months (new proposed 32 months)	0%	
Subtask 2: Perform histologic and serologic analysis after hydrogel and microneedle administration in rats	12 months (new proposed 32 months)	0%	
Subtask 3: Assess for an immunologic response to Dsup protein by evaluating for anti-Dsup antibodies in rats	12 months (new proposed 32 months)	0%	
Major Task 3: Determine the kinetics of Dsup expression in the urethra, bladder, and rectum after hydrogel and microneedle delivery			
Subtask 1: Measure the kinetics of Dsup protein expression after single administration for up to 14 days in rats	18 months (new proposed 32 months)	0%	
Major Task 4: Quantify the level of radioprotection of the Dsup mRNA-PBAE loaded hydrogels and microneedles in rats and pigs			
Subtask 1: Evaluate the degree of radioprotection conferred by Dsup-loaded hydrogels and microneedles in rats	20 months (new proposed 36 months)	0%	
Subtask 2: Evaluate the degree of radioprotection conferred by Dsup-loaded hydrogels and microneedles in pigs	20 months (new proposed 36 months)	0%	
Subtask 3: Assess the impact of multiple administrations on radioprotection in rats	24 months (new proposed 36 months)	0%	
Subtask 4: Evaluate the effect of radiation dose on radioprotection	24 months (new proposed 36 months)	0%	

3. Significant Results and Outcomes:

Major Task 1. **Develop methods for mucosal administration of Dsup mRNA.** We had previously generated and tested a library of poly beta(amino ester) (PBAE) polymers for nanoparticle transfection of enhanced green fluorescent protein (eGFP) mRNA and the damage suppressor (Dsup)-GFP fusion mRNA in human oral epithelial and colorectal cancer (Caco-2) cells. The Caco-2 cell line was used *in lieu* of primary cells due to the ease of use and lifetime of these cell lines. Upon attempting initial transfection studies in the rectum and oral mucosa of rats, we were unable to demonstrate successful transfection through direct injection of PBAE GFP mRNA nanoparticles into rectal and oral mucosa; the oral mucosa was performed due to ease of administration for microneedle platforms before transitioning to the rectum. The lack of *in vivo* transfection raised the concern about the stability of the PBAE mRNA nanoparticles in the presence of the tissue environment, as well as tissue specificity of the PBAE polymer. We, therefore, changed the formulation to incorporate cationic and non-ionic lipids with PBAE polymers to created PBAE-lipid nanoparticles. There are numerous pre-clinical and clinical examples of using lipids for nanoparticle transfection of mRNA (Hou X, et al. Nature Reviews Materials 2021). We performed initial characterization of the PBAE-lipid nanoparticles through dynamic light scattering and *in vitro* assays including high throughput assays in Caco-2 cells (**Figures 1 and 2**). Dynamic light scattering demonstrated a smaller nanoparticle with the addition of the PBAE polymer with lipids compared to the lipid nanoparticles alone (**Figure 2B**). The high throughput assay identified cell-type specificity for the different PBAE polymers **(Figure 1**), and we will move forward with the PBAE-lipid nanoparticle with the highest amount of transfection.

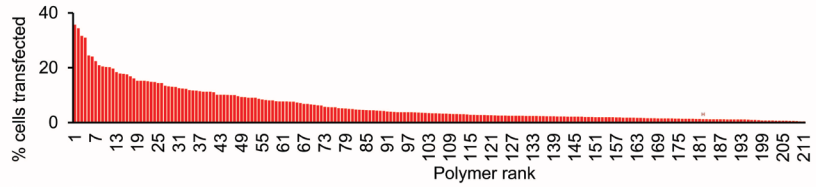
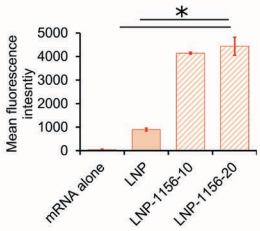


Figure 1. Repeat high throughput flow cytometry screen of 211 different PBAE polymers in combination with additional lipids (DOPE, cholesterol, DP2, and for transfection of GFP mRNA in Caco-2 cells.



Formulation	Particle size (nm)	Polydispersity index	Zeta potential (mV)
LNP	150 (2.2)	0.35 (0.01)	31.7 (0.8)
LNP-20-1156	126.4 (0.8)	0.17 (0.01)	30.9 (2.5)

Figure 2. Transfection of eGFP mRNA using the top PBAE polymer candidate (1156) in combination with lipids in Caco-2 cells (left) and the nanoparticle characteristics (right).

We have created a microneedle-based strategy to evaluate the delivery of nanoparticles *in vivo* (**Subtask 2**). The protocol for microneedle fabrication is critical given the lability of mRNA and the nanoparticles. We identified a rapid microneedle fabrication strategy without the use of heat and non-polar solvents. Using a 3D printed microneedle platform, we created a negative replica using polydimethylsiloxane (PDMS). Subsequently, using a polyvinylpyrrolidone-polyvinyl alcohol (9:1 ratio, 5wt% solution) was added to the negative replica, centrifuged, and allowed to dry for 30 minutes at room temperature. A polycaprolactone backing was applied at room temperature, allowing removal of the microneedles. **Figure 3** shows a rapidly dissolvable polyvinylpyrrolidone-polyvinyl alcohol microneedles that were administered into porcine buccal mucosa. Figure 3 shows the microneedles after 15 seconds (**Figure 3A**), 30 seconds (**Figure 3B**), and 45 seconds (**Figure 3C**), and the height loss of the microneedles is quantified in **Figure 3D**. This work builds upon the curved microneedles previously developed. (**Subtasks 4-6**). We continue to pursue hydrogel-based strategies for the ability to administer to the urothelium; however, the primary challenge of this strategy is particle concentration.

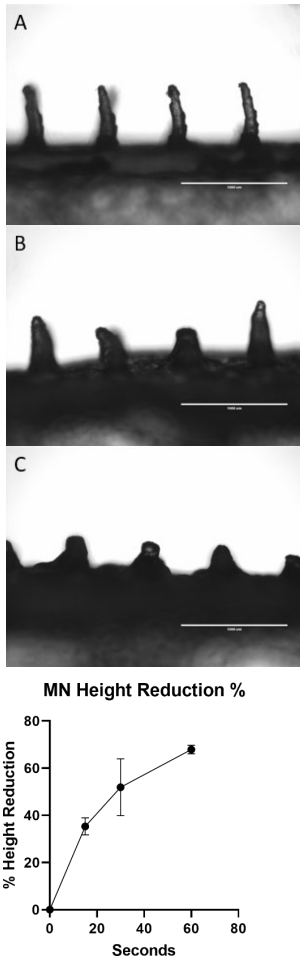


Figure 3. (A) Rapidly dissolvable PVP microneedles with Dsup-GFP mRNA-loaded PBAE particles. (B) Rhodamine-filled microneedles applied to ex vivo pig rectum.

Major Task 2. **Evaluate the safety of Dsup expression in a rat model.** After confirming a high degree of *in vitro* transfection, we performed direct injection of PBAE-lipid nanoparticles into the oral mucosa. We achieved successful *in vivo* transfection of luciferase mRNA through direct injection in the oral mucosa in rats (**Figure 4**), where luminescence was assessed at 3 and 6 hours, and the luminescence from PBAE-lipid nanoparticles was compared to lipid nanoparticle, mRNA alone, and untreated controls (n = 3). Luminescence was greater at 6 hours than 3 hours for the PBAE-lipid nanoparticles. Luciferase mRNA was used due to the ease of evaluation using an *in vivo* imager. There was no redness of the buccal mucosa, body score changes, or weight loss over 72 hours after administration. Additional testing is underway to evaluate rectal administration through direct injection, as well as reformulating the nanoparticles in microneedles and gels for administration. Safety will be assessed. We will be replicating these results in rectums of rats and pigs using the Dsup mRNA loaded PBAE

particles upon approval of the ACURO.

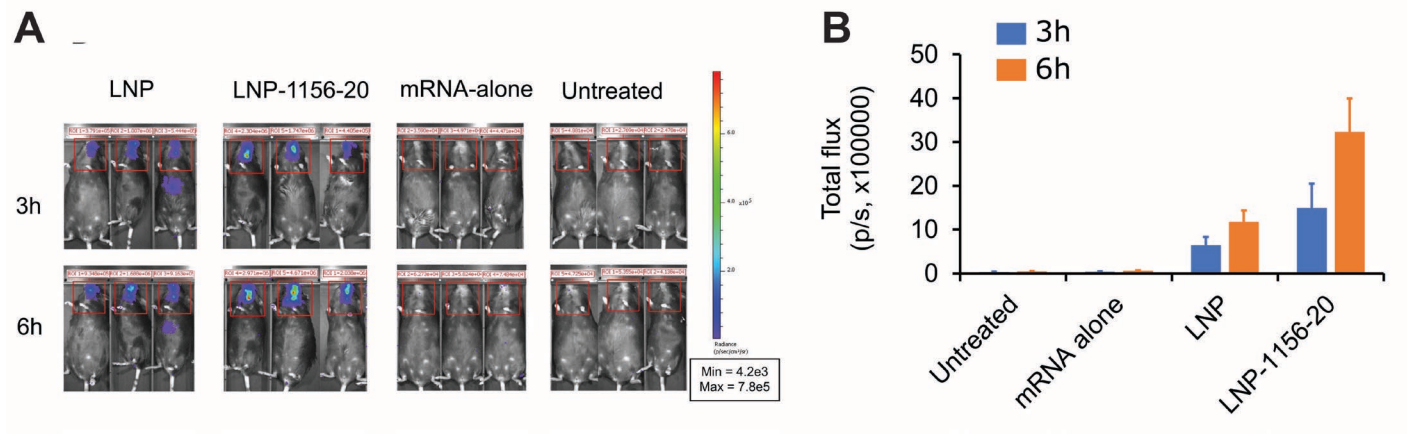


Figure 4. Luminescence imaging of human rectal adenocarcinoma cell transfected with Dsup-GFP mRNA.

Opportunities for training and professional development

Tasks and subtasks. Accomplishments are in *italics*.	Timeline	Percent completed	Accomplished
Major Task 1: Training and educational development in prostate cancer research			
Subtask 1: Attend scientific research workshops offered through MIT/BWH/DFCI to help develop a better understanding of drug and device development from design to commercialization. *PI attends workshops at BWH and MIT focused on translation of technologies.*	24 months	100%	X
Subtask 2: Participate in an educational curriculum that involves taking coursework "Innovation and Commercialization" at MIT and "Tumor Pathophysiology: A Systems Biology Approach" at Harvard Medical School. *PI was a project mentor and attendant for the course, MIT Medical Device Design (MIT 2.75) that involved innovation and commercialization at MIT.*	24 months	100%	X
Subtask 3: Attend and present research at Dr. Langer's (mentor) monthly research group meetings. PI *attends and will be presenting at Dr. Langer's research group meeting in summer 2021.*	24 months	100%	X
Subtask 4: Attend and present research at Dr. Traverso's (mentor) weekly research sub-group meetings. *PI attends and has presented at Dr. Traverso's research group meeting (8/2020, 11/2020, 3/2021, 5/2021).*	24 months	100%	X
Subtask 5: Receive weekly/monthly formal didactic/teaching sessions from Dr. D'Amico in applying methods from statistics and prostate cancer toward translational research. *PI attends monthly formal didactic/teaching sessions by Dr. D'Amico.*	24 months	100%	X
Subtask 6: Attend and present at national scientific meetings relevant to prostate cancer, engineering, and drug delivery. *PI has presented at the Prostate Cancer Foundation annual meeting 2019, 2020, and 2021.*	24 months	100%	X
Subtask 6: Prepare manuscripts relevant to radioprotectants and nucleic acid delivery to prevent radiation-induced toxicities in prostate cancer patients under the guidance of my mentors.	24 months	50%	

Results disseminated to communities of interest
The interim results were presented at the Prostate Cancer Foundation annual meeting.

Plan to do during the next report period
We will be completing studies evaluating the stability of the microneedles over 28 days (Major Task 1, Sub-task 6). Furthermore, we will be assessing hydrogels for urethral administration. will be evaluating and performing the safety, expression, and degree of radioprotection of the particle-loaded microneedles *in vivo* (Major Tasks 2-4).

4. IMPACT

Impact on the development of the principal disciplines of the project
This project has broad implications for treatments involving the local delivery of macromolecules and nucleic acids. Certain other localized diseases/conditions would benefit from such approaches, including skin disorders, ocular disease, and conditions that involve the GI mucosa. Furthermore, vaccine development through this approach may also be possible, as evidenced by recent mRNA vaccines by companies, Pfizer and Moderna.

Impact on other disciplines
This work may impact other disciplines outside of healthcare, including veterinary medicine, botany, and microbiology, through the delivery of mRNA. In addition, the use of the damage suppressor protein may be useful in bioremediation where having extremotolerant bacteria would be useful for the removal of radioactive and toxic chemicals.

Impact on technology transfer
Nothing to report

Impact on society beyond science and technology
Nothing to report

5. CHANGES/PROBLEMS

Changes in approach and reasons for change
The primary change in approach is a difference in formulation of the nanoparticles due to the lack of *in vivo* transfection using PBAE mRNA nanoparticles.

Actual or anticipated problems or delays and actions or plans to resolve them
There have been significant delays in the resubmission of the ACURO and *in vivo* studies as a result of moving institutions. It is expected that these activities will progress forward. The PI will maintain a position at Brigham and Women's Hospital to supervise this project, as there are certain materials and expertise that would only be available at BWH and MIT.

Changes that had a significant impact on expenditures
Expenditures slowed significantly during the move of institutions. As a result, expenditures were significantly delayed. Furthermore, the delay in ACURO resubmission halted research ordering within the PI's department.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to report

Significant changes in use or care of human subjects.
Nothing to report

Significant changes in use or care of vertebrate animals.
Nothing to report

Significant changes in use of biohazards and/or select agents
Nothing to report

6. PRODUCTS

Publications, conference papers, and presentations

- **Journal publications.**

  Young CC, Byrne JD, Wentworth AJ, Collins JE, Chu JN, Traverso G. Respirators in Healthcare: Material, Design, Regulatory, Environmental, and Economic Considerations for Clinical Efficacy. Global Challenges 2022; 2200001.

  Huang HW, You SS, Tizio LD, Li C, Raftery E, Ehmke C, Steiger C, Li J, Wentworth A, Ballinger I, Gwynne D, Nan K, Liang JY, Li J, Byrne JD, Collins J, Tamang S, Ishida K, Halperin F, Traverso G. An automated all-in-one system for carbohydrate tracking, glucose monitoring, and insulin delivery. Journal of Controlled Release. 2022; 343:31-42.

  Chu LM, Shaefi S, Byrne JD, Alves de Souza RW, Otterbein LE. Carbon Monoxide and a Change of Heart. Redox Biology. 2021; 48: 102183.

  Chu JN, Collins J, Chen T, Chai P, Dadabhoy F, Byrne JD, Wentworth A, DeAndrea-Lazarus I, Moreland C, Wilson J, Booth A, Ghenand O, Hur C, Traverso C. Patient and Health Care Worker Perceptions of Communication and Ability to Identify Emotion When Wearing Standard and Transparent Masks. JAMA Network Open. 2021; 4 (11): e2135386.

- **Books or other non-periodical, one-time publications.** Nothing to report

- **Other publications, conference papers, and presentations.** Nothing to report

Website(s) or other Internet site(s)
Nothing to report

Technologies or techniques
Nothing to report

Inventions, patent applications, and/or licenses
Traverso CG, Kirtane AR, Langer RS, Kim H, Liu GW, Byrne JD, Reker D, Shi Y, Zhong GJ, Rajesh N. Branched poly(-amino esters) for the delivery of nucleic acids. PCT filed March 8, 2022.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	*James Byrne*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	10
Contribution to Project:	*Dr. Byrne performed experiments involving tardigrade-unique mRNA delivery*
Funding Support:	*Prostate Cancer Foundation, Holden Comprehensive Cancer Center*

Name:	*Jianling Bi*
Project Role:	Postdoctoral fellow
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	2
Contribution to Project:	*Dr. Bi assisted with in vitro evaluation of PBAE particles*
Funding Support:	*Holden Comprehensive Cancer Center start-up package*

Name:	*Sarah Becker*
Project Role:	Research Technician
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	2
Contribution to Project:	*Ms. Becker assisted with in vitro evaluation of PBAE particles*
Funding Support:	*Prostate Cancer Foundation*

Name:	*Ameya Kirtane*
Project Role:	Collaborator, Instructor
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3
Contribution to Project:	*Dr. Kirtane synthesized the PBAE polymers and assisted with nanoparticle fabrication and testing*
Funding Support:	*Gates Foundation*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

University of Iowa Hospitals and Clinics, 2021-present

Salary support PI: James Byrne

What other organizations were involved as partners?

- **Organization Name:** MIT, University of Iowa
- **Location of Organization:** *Cambridge, MA; Iowa City, IA*
- **Partner's contribution to the projec**t
  - **Facilities**: Animal facilities
  - **Equipment**: Nanoparticle fabrication and analysis

8. SPECIAL REPORTING REQUIREMENTS

Nothing to report

9. APPENDICES

Nothing to report