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TITLE: Extremophile RNA Delivery for Radioprotection in Prostate Cancer Patients

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CONTRACTING ORGANIZATION: Brigham and Women's Hospital
Boston, MA

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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	87.5%	
Major Task 2: Evaluate the safety of Dsup expression in a rat model	12 months (new proposed 18 months)	0%	
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 have synthesized 211 different PBAE polymers to test the delivery of mRNA. These polymers selectively transfect certain cells. In addition, we have identified top polymers candidates for transfection of mRNA in Caco-2 cells using high throughput flow cytometry screens. This cell line was used in lieu of primary cells due to the ease of use and lifetime of these cell lines. We have demonstrated a significant reduction in DNA damage to a single dose of 10 Gy of radiation in cells that express the Dsup protein (Comet assay and immunofluorescence for phosphorylated gamma-H2AX). Furthermore, we have demonstrated an improvement in cell viability after exposure to a single dose of 10 Gy. We have also created clinically relevant materials for *in vivo* application. Rapidly dissolvable microneedles loaded with PBAE nanoparticles have been created and tested in vitro for post-processing function. We will begin *in vivo* evaluation of these materials upon approval of the ACURO.

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 10 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 18 months)	0%	
Subtask 2: Perform histologic and serologic analysis after hydrogel and microneedle administration in rats	12 months (new proposed 18 months)	0%	
Subtask 3: Assess for an immunologic response to Dsup protein by evaluating for anti-Dsup antibodies in rats	12 months (new proposed 18 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	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	0%	
Subtask 2: Evaluate the degree of radioprotection conferred by Dsup-loaded hydrogels and microneedles in pigs	20 months	0%	
Subtask 3: Assess the impact of multiple administrations on radioprotection in rats	24 months	0%	
Subtask 4: Evaluate the effect of radiation dose on radioprotection	24 months	0%	

3. Significant Results and Outcomes:

Major Task 1. **Develop methods for mucosal administration of Dsup mRNA.** Given the cell selectivity of PBAE polymers for transfection, we synthetized 211 different PBAE polymers. High throughput flow cytometry screens of 211 different PBAE polymers for transfection of green fluorescent protein (GFP) mRNA in human colorectal adenocarcinoma cells (Caco-2 cells) enabled the identification of the top 10 polymer candidates (teal color) (**Subtask 1**). Polymer numbers correspond to different PBAE polymers with unique side chains. Furthermore, Figure 1 showcases representative images of the GFP expression among the PBAE candidates enabling the highest and lowest degrees of transfection. Upon identifying these polymer candidates, we next generated a fusion mRNA coding for the damage-suppressor protein (Dsup) and GFP. Using the top 10 polymer candidates, we performed another high throughput flow cytometry screen using the Dsup-GFP fusion mRNA in human oral epithelial and Caco2 cells (Figure 2A and B). There were multiple polymers that enabled high transfection efficiency up to 60% of human oral epithelial cells and 40% in Caco-2 cells. The next step for translation of this system was to verify the functionality of the Dsup protein in protecting DNA from the damaging effects of radiation. Using the top polymer candidate, 856, we transfected human Caco-2 cells and verified the nuclear localization of the Dsup-GFP fusion protein after translation of the delivered mRNA.

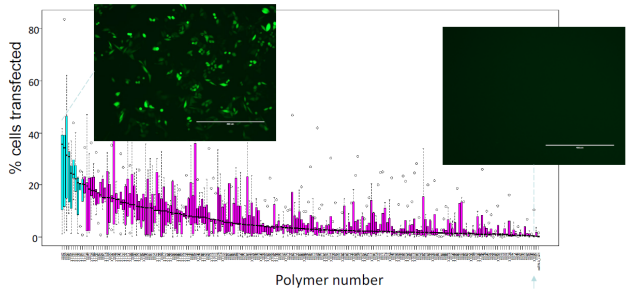

Figure 1. High throughput flow cytometry screen of 211 different PBAE polymers for transfection of GFP mRNA in Caco-2 cells. The top 10 polymer candidates are identified by the teal color.

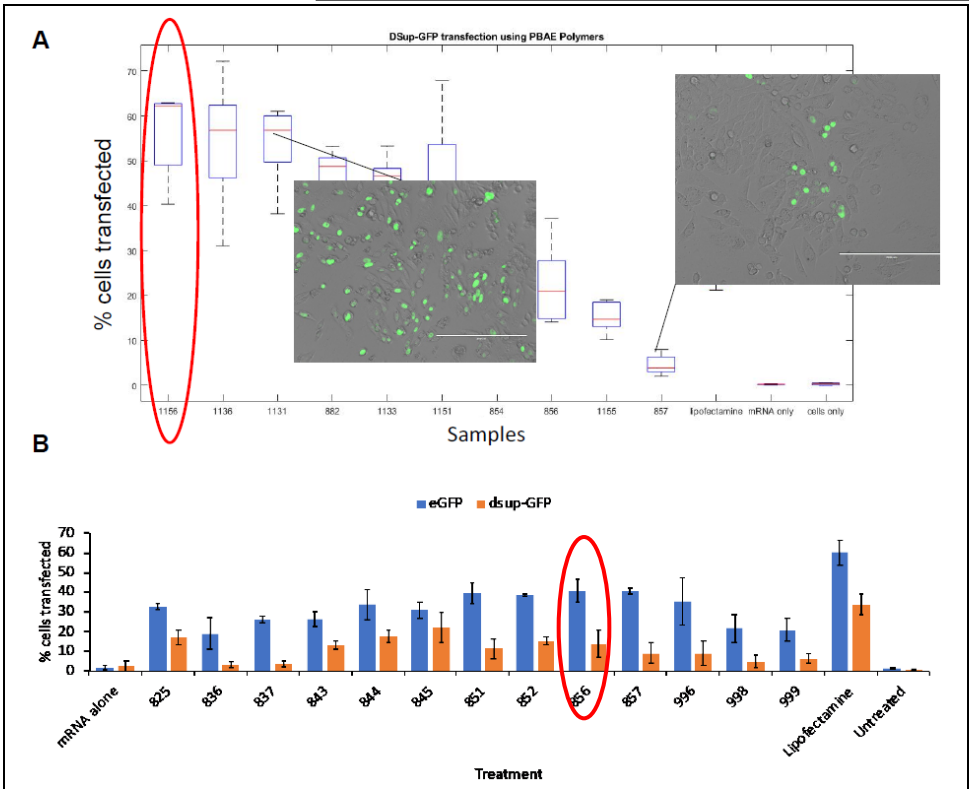

Figure 2. High throughput flow cytometry screen of 10 different PBAE polymers for transfection of Dsup-GFP mRNA in (A) human oral epithelial cells and (B) Caco-2 cells. Transfection efficiency with the top polymer candidates was up to 60%.

We have created a microneedle-based strategy to evaluate the functionality of the particles in vivo (**Subtask 2**). Figure 3A shows a rapidly dissolvable polyvinylpyrrolidone microneedles with Dsup-GFP mRNA-loaded PBAE particles loaded in. Due to the challenge of penetration in the highly compressible and elastic surface of the rectum, we have created curved microneedles to enable improved delivery. In addition, we showcase the penetration of curve microneedles into *ex vivo* rectal tissue of pigs (Figure 3B). We are currently assessing unique rectal applicators for the administration of curved microneedles. Furthermore, we have validated the *in vitro* expression of the PBAE particles after 1 days from initial fabrication (Figure 4) (**Subtasks 4-6**). We continue to pursue hydrogel-

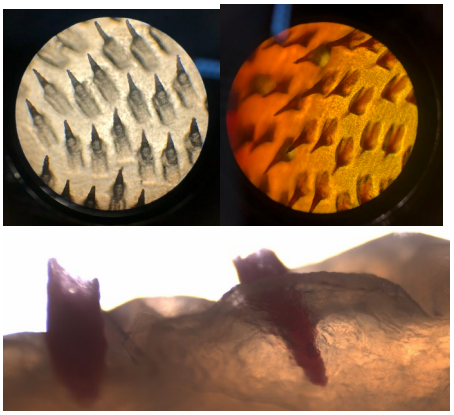

Figure 3. (A) Rapidly dissolvable PVP microneedles with Dsup-GFP mRNA-loaded PBAE particles. (B) Rhodamine-filled microneedles applied to ex vivo pig rectum.

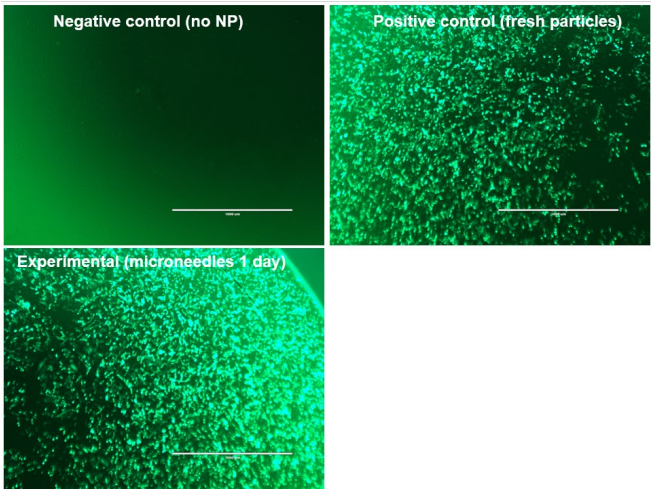

Figure 4. In vitro confirmation of particle stability in microneedles after 1 day. Particles are suspended in PVP microneedles and maintained under desiccant.

based strategies for the ability to administer to the urothelium; however, the primary challenge of this strategy is particle concentration.

Major Task 2. Evaluate the safety of Dsup expression in a rat model. As an initial step towards in vivo evaluation, we performed direct injection of mRNA encoding Cre recombinase encapsulated in PBAE nanoparticles into the gastric wall of rodents and pigs (not funded by this award). Here, we showcase the expression of a Cre recombinase in rats and pigs after injection (Figure 5). We found that there was no significant gross inflammation as a result of a single injection. Furthermore, histological assessment of H&E stained tissues demonstrated no significant increase in cellular infiltrate within the gastric wall. We will be replicating these results in rectums of rats and pigs using the Dsup mRNA loaded PBAE particles upon approval of the ACURO.

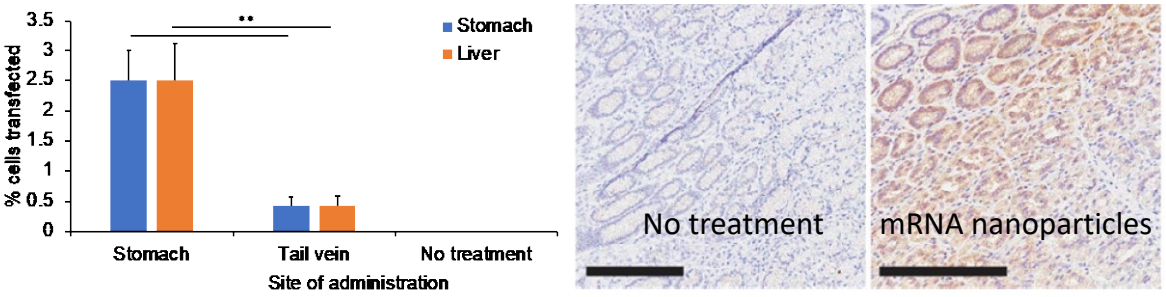

Figure 5. (A) Rodents were treated with mRNA encoding Cre recombinase encapsulated in PBAE nanoparticles via a stomach or tail vein injection. Rodents were sacrificed and expression of tdTomato in the stomachs and livers of the animals were measured using flow cytometry. Stomach injections led to higher transfection efficiency in the stomach and liver compared to tail vein injection. **p<0.01, one-way ANOVA, post-hoc Bonferroni. (B) Pigs were treated with mRNA encoding Cre recombinase encapsulated in PBAE nanoparticles via stomach injection. A biopsy of the stomach was obtained from untreated animals and mRNA nanoparticle treated animals. Tissue sections were obtained and stained for expression of Cre recombinase.

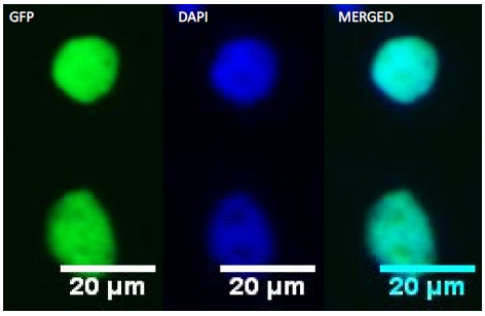

Figure 6. Fluorescence imaging of human rectal adenocarcinoma cell transfected with Dsup-GFP mRNA.

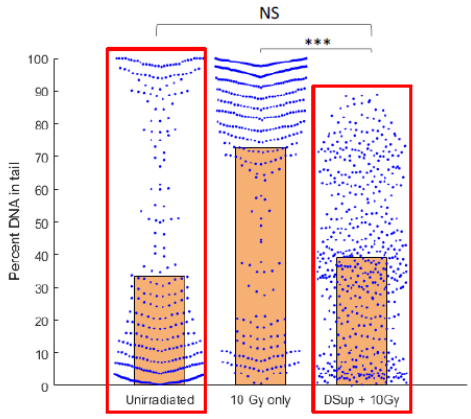

Figure 7. Delivery of Dsup-GFP mRNA results in significantly reduced single stranded DNA breaks.

4. Other Achievements:

Through nuclear staining (DAPI) of transfected cells, we validated that the Dsup-GFP fusion protein was in the nucleus of the cells where it can bind to DNA (Figure 6). To evaluate the degree of protection from radiation-induced DNA damage, we performed two assays commonly used in radiation biology, including a Comet assay and immunofluorescence staining for phosphorylated gamma-H2AX. The Comet assay involves the electrophoresis of single cells in order to detect DNA damage and its repair. Cells are exposed to ionizing radiation, embedded in agarose, and then subjected to an electrical gradient to move the DNA into the gel. The negatively charged DNA in the cell moves through the agarose toward the positive electric pole. If there are no breaks, the cell's DNA moves all together in a small ball. Double-strand DNA breaks creates DNA fragments that are smaller than the unbroken DNA and migrate further into the agarose making what appears like a comet's tail. Alkaline conditions cause the separation of the two strands of the DNA helix and allows the visualization of DNA fragments created by both double-strand and single-strand DNA

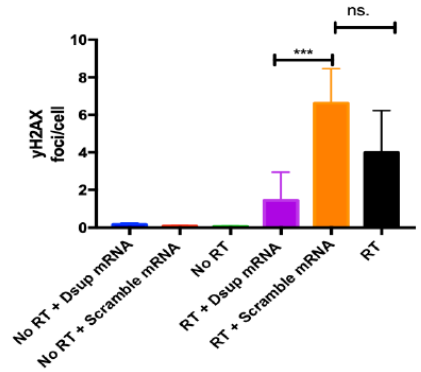

Figure 8. Immunofluorescence staining phosphorylated gamma-H2AX in human oral epithelial 30 minutes after exposure to a single 10 Gy dose of radiation.

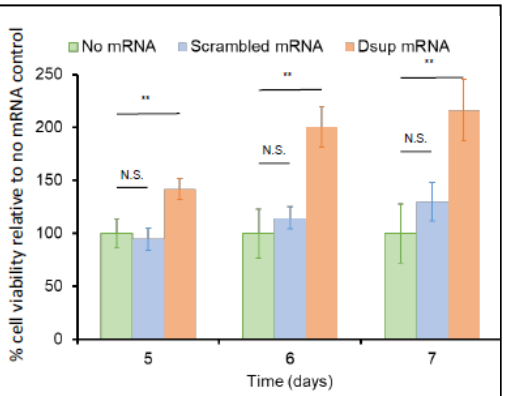

Figure 9. Cell viability in human oral epithelial cells exposed to a single 10 Gy dose of radiation. Cell viability was determined using CellTiter Glo.

breaks. We performed an alkaline Comet assay on our Dsup-GFP fusion mRNA transfected cells after exposure to a single 10 Gy dose of radiation (Figure 7).

Compared to cells that were not transfected, the transfected cells had significantly less single strand DNA breaks (noted to be percent DNA in tail). Furthermore, the amount of single strand DNA breaks in the cells that were transfected and treated with 10 Gy of radiation was similar to unirradiated, non-transfected cells. To further evaluate the degree of radiation protection afforded by the expression of Dsup-GFP fusion protein, we performed immunofluorescence staining for phosphorylated gamma-H2AX after exposure to radiation. The production of DNA double-strand breaks (DSBs) by ionizing radiation leads to the rapid phosphorylation of histone H2AX on serine 139 (gamma-H2AX). The specificity of this reaction provides a reliable yardstick. We exposed transfected cells to a single dose of 10 Gy of radiation and then stained for identification of the phosphorylated gamma-H2AX. We identified a significant reduction in phosphorylated gamma-H2AX for cells that were transfected compared to non-transfected cells and cells treated with a scramble mRNA (Figure 8). We evaluated the impact of Dsup expression on cell viability after exposure to radiation. We demonstrated that the Dsup protein significantly improved cell viability after exposure to a single dose of 10 Gy of radiation (Figure 9). Given that the effects of radiation are normally seen on subsequent generations of cells, these results validate that the Dsup protein enables a high degree of protection whilst also allowing the cells to continue dividing and proliferating.

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	50%	
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	50%	
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	50%	
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	50%	
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 and 2020.*	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	0%	

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 he 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
There have been no changes in the approach.

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. Delays are a result of unanticipated COVID-related changes to research within the department and hospitals where the PI works. As the research facilities have opened up, it is anticipated that these activities will progress forward. Furthermore, the PI will be moving institutions from Brigham and Women's Hospital for a position at the University of Iowa this summer. He 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 pandemic and research stoppage. 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.** Nothing to report
- **Books or other non-periodical, one-time publications.** Nothing to report
- **Other publications, conference papers, and presentations.** Byrne JD, et al. Prostate Cancer Foundation Annual Meeting 2020. Poster presentation.

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

Technologies or techniques
Nothing to report

Inventions, patent applications, and/or licenses
Nothing to report

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:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support:	*The Ford Foundation (Complete only if the funding support is provided from other than this award).*

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?

Hope Funds for Cancer Research Post-Doctoral Fellowship, 2020-2023
per year (salary support) PI: James Byrne
MIT Mentor: Robert Langer

What other organizations were involved as partners?

- **Organization Name:** MIT
- **Location of Organization:** *Cambridge, MA*
- **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